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(54) Title: A METHOD FOR THE ISOLATION AND PURIFICATION OF LIPID CELL-WALL COMPONENTS

(57) Abstract

A method, using a multi-phasic solvent system, for the simultaneous purification and separation of different classes of compounds, which may be cell-wall components or derivatives or analogues thereof which may be extracted from a culture of the relevant cells or which may be synthesized. The cell-wall components may be lipid cell-wall components of microbial origin which can be separated from contaminating material as a group.

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A METHOD FOR THE ISOLATION AND PURIFICATION OF LIPID CELL-WALL COMPONENTS

BACKGROUND OF THE INVENTION

This invention relates to the isolation and purification of lipid cell-wall components originating from bacteria assigned to the genera ***Mycobacterium***, ***Corynebacterium***, ***Nocardia*** or ***Rhodococcus***, of which the most ubiquitous and the most important from a human health point of view is the genus ***Mycobacterium***.

The genus ***Mycobacterium*** comprises a large number of both saprophytic and pathogenic species. The best known members of the genus, *M. tuberculosis* and *M. leprae*, are the causative agents of tuberculosis and leprosy, respectively, both among the most serious diseases occurring in man.

Tuberculosis: Current status

Tuberculosis is considered to be the major communicable disease throughout most of the world. Despite great advances in medical science and a range of effective drugs, which for some time created the impression that the disease had been conquered, and despite organised international efforts, tuberculosis remains a world health problem of staggering proportions: approximately one third of the world's population is infected with *M. tuberculosis* (Fauci, 1995), more than 8 million new cases world-wide and more than 3 million deaths in the year 1990 alone were reported (Snider, 1994). Predictions made by the World Health Organisation indicate that by the year 2000 the annual figures will grow to 10,2 million new cases and 3,5 million deaths, with Asia and sub-Saharan Africa being the most affected continents (De Cock et *al.*, 1992; Dolin, Raviglione and Kochi, 1994; Raviglione, Snider and Kochi, 1995; Wilkinson and de Cock, 1996). According to the recently released "WHO Report on the Tuberculosis Epidemic, 1995" the figures anticipated for the next decade are even more alarming: 300 million new infections and 30 million deaths (Holler, 1995). In effect, tuberculosis was declared in 1993 by WHO to be a global public health emergency (Bloomfield, 1995; Wilkinson and de Cock, 1996).

The major reasons for this dramatic comeback and the unabated spread of tuberculosis can be identified as:

1) Insufficient protection offered by the world-wide vaccination programme based on the use of BCG*)

*) **BCG:** (Bacillus of Calmette and Guerin) Calmette and Guerin attenuated a strain of *M. bovis* by passaging it 231 times over a period of 13 years through a medium containing glycerine and oxbile.

2) Problems associated with the detection of tuberculosis

3) Problems associated with treatment of tuberculosis and the occurrence of multi-drug resistant strains of *M. tuberculosis*

4) Interaction with HIV infection

5) Socio-economic aspects

1. Insufficient protection offered by the world-wide vaccination programme based on BCG

Attempts to prevent the spread of tuberculosis by inducing resistance to the infection with *M. tuberculosis* were initiated at the beginning of this century, using vaccination with BCG. On the basis of a number of controlled studies it was established that the protective efficacy obtained in vaccination with BCG varied between 0 to 80% (Snider, 1994; Hershfield, 1995) and, on the basis of the analysis of the published literature, the BCG vaccination was found to be roughly 50% effective (Colditz *et al.*, 1994; O'Brien, 1995). A number of hypotheses/explanations for this unsatisfactory situation have been put forward (Fine, 1994). The most important are:

i) Variations between BCG vaccines, which could be caused by strains variation or by differences between manufacturing processes;

ii) Differences in pathogenesis of *M. tuberculosis*;

iii) Differences in the exposure to the environmental mycobacteria - the environmental mycobacteria may act antagonistically or synergistically with BCG;

iv) Genetic differences between population groups subjected to vaccination with BCG;

v) Differences in nutrition and exposure to sunlight between various population groups;

vi) Differences between designs of various studies;

vii) Inadequacies of the criteria used for the evaluation of protective action of vaccination with BCG.

2. Problems associated with the detection of tuberculosis

The accurate and timely detection of tuberculosis and related mycobacterial diseases is one of the important requirements for the development of a more successful global strategy to combat these diseases.

Traditional laboratory detection methods have major disadvantages of either not being capable of distinguishing between live and dead bacilli (the quick and simple Ziehl-Neelsen staining) or, if these methods confirm the presence of the live bacilli (direct cultivation), a number of weeks is required before the laboratory tests are completed. This in turn, may delay the commencement of treatment and may lead to further spread of the disease.

Although recently developed molecular approaches to the diagnosis of tuberculosis (Godfrey-Faussett, 1994; Richeldi, Barnini and Saltini, 1995; Bloomfield, 1995; Vlaspolder, Singer and Roggeveen, 1995) resulted in the introduction of these rapid and sensitive detection tools by advanced laboratories in the developed countries, they are expensive and require specially trained personnel. For these reasons they are not suitable for screening/detection of tuberculosis in resource-poor, TB-endemic regions, already overburdened with the cost of controlling the disease (O'Brien, 1995; Voelker, 1995).

A similar situation exists in the field of rapid drug sensitivity testing (Schaberg *et al.*, 1995; Pretorius *et al.*, 1996) and rapid culturing of *Mycobacterium* (Bloomfield, 1995). The significant advances in these areas cannot be utilised, for financial reasons, in the countries most affected by the tuberculosis pandemic.

3. Problems associated with treatment of tuberculosis and the occurrence of multi-drug resistant strains of *M. tuberculosis*

The development of effective chemotherapy for tuberculosis made the treatment of infected persons possible, thus preventing the full development of the disease. Although the anti-tuberculosis drugs with proven bactericidal action (rifampicin, isoniazid and pyrazinamide) as well as the ones with bacteriostatic or resistance-preventing properties (streptomycin sulphate, ethambutol and thiacetazone) are available (Weil, 1994), world-wide success in combating the disease has not been achieved so far due to two main factors: patients' non-compliance with the prescribed regimen and financial limitations existing in developing countries.

The impact of the isoniazid preventative therapy on the control of tuberculosis in developing countries is uncertain. This approach, although widely practiced in North America, has two major disadvantages. Firstly, it may have to be given for the duration of the TB sufferers' life and, secondly, its cost, *i.e.* US$18 per patient per 6-month course may be prohibitive, particularly in the most affected areas where an amount of US$4 per patient per year is available for total health care (O'Brien, 1995).

The interrupted and/or uncompleted treatment, apart from the detrimental effects on the individual concerned, has contributed to the emergence and spread of multi-drug resistant strains of *M. tuberculosis,* which further complicate the overall situation (Beyers *et al.*, 1996). The recent WHO estimates indicate that 50 million people worldwide may already be carrying strains of *M. tuberculosis* resistant to one or more of the most common anti-TB drugs. It was established already in 1991, that one third of all TB patients in New York were resistant to at least one drug and almost 20% were resistant to rifampicin and isoniazid combined (Henderson 1995).

4. Interaction with HIV infection

The close association documented between tuberculosis and HIV infection as well as the frequently concomitant presence of both these diseases add gravity to the situation (Torres *et al.*, 1990; De Cock, 1994; Cantwell and Binkin, 1994; Murray, 1994; Antonucci *et al.*. 1995; Mofeson *et al.*, 1995; Davies, Wilkinson and Colvin, 1996; Wilkinson and Moore, 1996). The number of people who develop tuberculosis in Asia because of the parallel infection with the HIV is expected to increase seven-fold this decade, according to the Public Health Reports (1995).

The emergence of multiple-drug resistance among the strains of *M. tuberculosis* and other atypical mycobacteria has introduced an additional dimension to this problem (Blumberg, Miller and Koornhof, 1994; Morse, 1994; Yew and Chau, 1995).

5. Socio-economic aspects

Other reasons contributing to a further spread of the disease, such as unemployment, overcrowding, general lowering of economic conditions, alcoholism and erosion of the public health infrastructure have been recently reviewed by Darbyshire (1995), Fauci (1995), Law *et al.*, (1995) and Mangtani *et al.*, (1995).

The increased influx of immigrants from the endemic areas to countries where tuberculosis has been well under control, *e.g.* United States of America, creates additional problems in containing the spread of tuberculosis (Huebner and Castro, 1995). The incidence of tuberculosis among immigrants to the USA is reported to be 12 times higher than that observed among the native-born population (Ballew and Becker, 1995).

The trends discussed above place an overwhelming pressure on the improvement of existing approaches to case-management of TB patients both in endemic areas and developing countries and on the development of new drugs capable of preventing and/or combating tuberculosis (Cole, 1995; Voelker, 1995). Although cautious optimism can be detected among the researchers involved in both fields (Mwinga, 1995; Grosset, 1995), currently prevailing economic restrictions, even in the developed countries, place serious limitations on funding required to develop new anti-tuberculosis drugs, the cost of which is estimated at US$150 million for

each new compound (Grosset, 1995).

Immunological prospects for prevention and treatment

In view of the seriousness of the problems listed above, the limitations associated with the existing methods of combating tuberculosis and the high costs of developing new forms of chemoprophylaxis and chemotherapy, immunological approaches could provide a relevant and realistic alternative to finding an effective and economically affordable solution to the control and treatment of tuberculosis and associated conditions.

Results obtained from a number of trials using treatment with killed cells of *M. vaccae* (Stanford and Grange, 1994) suggest that this saprophytic microorganism may find application in immunotherapy of tuberculosis either as a single agent (Bahr *et al.*, 1990a; Bahr *et al.*, 1990b; Stanford *et al.*, 1990a) or in conjunction with chemotherapy (Stanford *et al.*, 1990b; Prior *et al.*, 1995; Onybebujoh *et al.*, 1995).

The modulation of inflammatory processes attributed to the use of thalidomide has recently been utilised in combating tuberculosis. The beneficial effects of thalidomide on the clinical manifestation of tuberculosis reported by Cole (1995) provide grounds for considering the immunomodulatory properties of this drug in the treatment of the disease. These effects of thalidomide are attributed to its potent inhibitory action on tumour necrotic factor, a cytokine involved in the inflammatory processes accompanying tuberculosis.

The advantages stemming from these two approaches may be extended to

treatment of drug-resistant forms of tuberculosis. Encouraging data have been reported in this respect from a number of trials undertaken in Iran (Etemadi, Farid and Stanford, 1992) and in Kuwait, Rumania, Vietnam and India (Stanford and Grange, 1993).

Immunological targets

Attempts at identification of immunologically active components of *M. tuberculosis* over the last decades focused mainly on proteins (Daniel, 1984; Chaparas, 1984; Yanez *et al.*, 1986; Deshpande *et al.*, 1994; Torres *et al.*, 1994), polysaccharides (Daniel, 1984; Misaki, Azuma and Yamamura, 1987), peptidoglycolipids and phospholipids (Brennan, 1984), lipopolysaccharides (Hunter, Gaylord and Brennan, 1986), glycolipids (Brennan, 1984; Mc Neil *et al.*, 1989) and lipoarabinomannan (Arya, 1993; Sieling *et al.*, 1995).

Although lipid cell-wall components have been associated with the virulence of *M. tuberculosis* (Collins, 1994), these compounds, being of lipid nature, *i.e.* ß-hydroxy fatty acids, have been considered not to possess immunogenic properties. The possibility that mycolic acids might play a significant role in the complex process of immunological response/s of the human body to the infection with *M. tuberculosis* has become apparent only recently.

A humoral response to a mycolic acids-BSA conjugate was first observed in 1994 (South African Patent Application No 95/3077 and PCT Patent Application No. WO 95/28642). At about the same time, a measurable cellular immune response to mycolic acids was demonstrated by Beckman

et al., (1994). The authors discovered that these compounds stimulated proliferation of a rare subset of human double-negative T-cells and described a new way of antigen presentation by CD1 molecules occurring on professional antigen-presenting cells like macrophages and dendritic cells (Beckman *et al.*, 1994; Beckman *et al.*, 1995; Rosat *et al.*, 1995). Likewise, prenyl pyrophosphate was also found to be presented by CD1 molecules on antigen-presenting cells (Morita *et al.*, 1995).

Lipid cell-wall components of mycobacteria

The mycobacterial cell wall is considered to be a highly differentiated and complex structure, characterised by a very high content of lipids, which constitute about 60% of the cell wall mass (Grange, 1988). Its diagrammatic presentation is given in Figure 1.

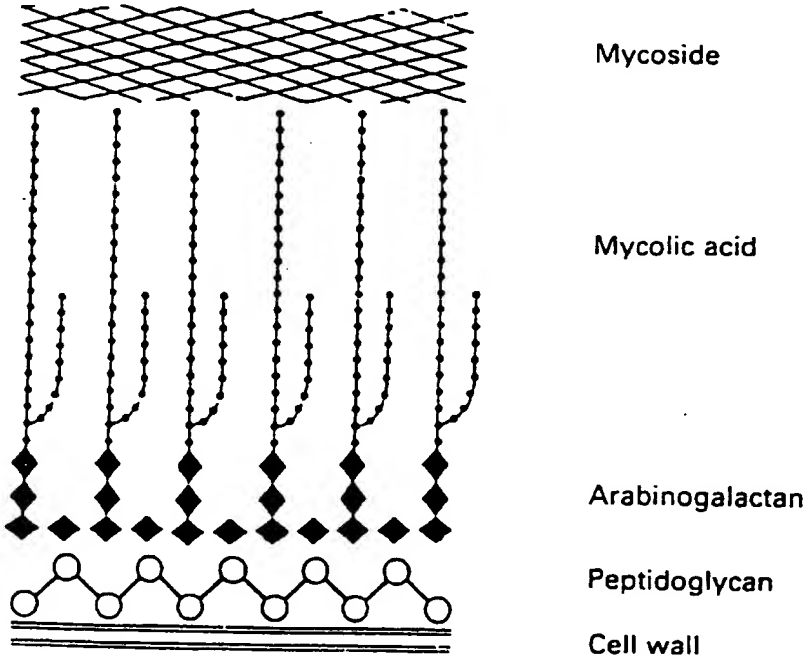


Fig. 1 Diagrammatic presentation of the mycobacterial cell wall

Mycolic acids, the major lipids of the cell wall of *Mycobacteria* (Petit and Lederer, 1984), are considered to be a major component of an outer permeability barrier (Wheeler *et al.*, 1994) and are responsible for the "acid-fastness" of this group of microorganisms (Grange, 1988). The presence of large amounts of mycolic acids associated with different types of free lipids constitutes the basis of the integrity of the mycobacterial wall (Besra *et al.*, 1993).

Mycolic acids occur in nature as mixtures of different types. They frequently form esters with carbohydrates, e.g. with arabinose forming the main cell wall palisade and with trehalose forming dimycolyl trehalose, the so called cord factor which is associated with the virulence of *M. tuberculosis*. Mycolic acids have been reported in bacterial species other than *Mycobacterium*, i.e. in *Corynebacterium* and *Nocardia* (Goren, 1972). Consequently, three major categories of mycolic acids are distinguished (The Merck Index, 1989), namely:

i) corynomycolic acids (C_{28} - C_{40} acyl chain length)

ii) nocardomycolic acids (C_{40} - C_{60} acyl chain length)

iii) mycobacterial mycolic acids (C_{60} - C_{90} acyl chain length).

Mycolic acids are high molecular weight ß-hydroxy fatty acids which have moderately long aliphatic chains at the α-position. The general formula of these compounds is shown in Figure 2.

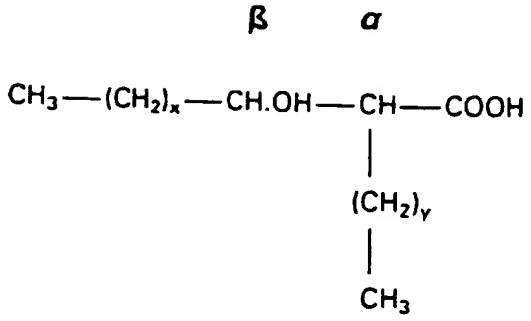


Fig.2 The general formula of mycolic acids

All known mycolic acids have the basic structure $R^2CH(OH)CHR^1COOH$, where R^1 is a C_{20} to C_{24} linear alkane and R^2 is a more complex structure of 30 to 60 carbon atoms that may contain various numbers of carbon-carbon double bonds and/or cyclopropane rings, methyl branches or oxygen functions such as C=O, $CH_3OCH=$,COOH (The Merck Index, 1989). Although there exists a great variety of mycolic acids, the α branch, except for length, is essentially invariant/constant in any group of mycolic acids (Goren, 1972).

Mycolic acids are soluble in a very limited range of solvents, which complicates their purification (Brennan and Nikaido, 1995) and leads to tedious and costly protocols. Beckman et al., (1994), for example, achieved purification of mycolic acids from *M.tuberculosis* only after derivatization with para-bromophenacylbromide, reversed phase HPLC separation, collection of the mycolic acids peak cluster fraction, resaponification and extraction.

Anticipating the potential role which lipid cell-wall components, and in particular mycolic acids, can play in immunotherapy and immunoprevention of tuberculosis and/or its side effects, a new, more efficient method of purifying large quantities of these components is described in this invention.

SUMMARY OF THE INVENTION

According to the invention a method of separating and purifying a specific microbial cell-wall component or a derivative or analogue thereof from an extracted mixture of the cell-wall component or derivative of analogue thereof and contaminants or from a synthetic mixture of the cell-wall component or analogue or derivative thereof and contaminants comprises the steps of:

dissolving the extracted mixture or synthetic mixture in a multi-phasic solvent to form a solution; and

subjecting the solution to liquid-liquid phase extraction.

The specific cell-wall component or derivative or analogue thereof may be a lipid or a sugar.

The specific cell-wall component or analogue or derivative thereof is preferably a lipid.

More preferably, the lipid is a fatty acid.

More preferably, the lipid is a mycolic acid.

More preferably still, the cell-wall component is a group of components or analogues or derivatives thereof and the group is a mixture of mycolic acids or derivatives or analogues thereof.

The microbial cell-wall component may be derived from a bacterium, a fungus or a yeast.

The microbial cell-wall component is preferably derived from a bacterium which may be selected from *Mycobacteria*, *Corynebacteria*, *Nocardia*, *Rhodococci*, *Amycolata* and other suitable bacterial species.

When the bacterium is selected from *Mycobacteria*, it may be selected from the strains of *M tuberculosis*, *M.avium* and *M.vaccae*.

The multi-phasic solvent system preferably comprises chloroform, methanol and water.

More preferably, the multi-phasic solvent system is a bi-phasic solvent system.

The bi-phasic solvent system preferably comprises an upper liquid phase and a lower liquid phase.

The method preferably also comprises the steps of mixing and equilibrating the upper and lower phases of the solvent system.

Preferably, the composition of the upper phase is 12-18% chloroform, 45-55% methanol and 25-40% water. More preferably, the composition of the upper phase is 15% chloroform, 52% methanol and 33% water.

Preferably, the composition of the lower phase is 50-80% chloroform, 15-40% methanol and 2-8% water. More preferably, the composition of the lower phase is 68% chloroform, 27% methanol and 5% water.

The liquid-liquid phase extraction may be a countercurrent extraction or a multiple extraction of one of the phases.

The purified cell-wall component or analogue or derivative thereof may be subjected to an acetone extraction to remove impurities.

Preferably, the cell-wall component or analogue or derivative thereof needs no chemical derivatisation to separate it from any impurities which may arise from microbial growth, microbial-growth media or the synthetic mixture.

The purified cell-wall component or derivative or analogue thereof may be saponified to reverse any methyl esterification thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in more detail, by way of example only, with reference to the accompanying drawings in which:

Graph 1a: is an HPLC of a crude *M. tuberculosis* extract

Graph 1b: is an HPLC of a crude reagents extract

Graph 1c: is an HPLC of a crude medium extract

Graph 2a: is an HPLC of an acetone extracted supernatant of the crude *M. tuberculosis* extract

Graph 2b: is an HPLC of an acetone extracted supernatant of the crude reagents extract

Graph 2c: is an HPLC of an acetone extracted supernatant of the crude medium extract

Graph 3a: is an HPLC of a crude *M. tuberculosis* extract, acetone extracted

Graph 3b: is an HPLC of a crude reagents extract, acetone extracted

Graph 3c: is an HPLC of a crude medium extract, acetone extracted

Graph 4a: is an HPLC of countercurrent purified mycolic acids of *M. tuberculosis,* from acetone extracted crude extract

Graph 4b: is an HPLC of countercurrent purified reagents from acetone extracted crude extract

Graph 4c: is an HPLC of countercurrent purified medium from acetone extracted crude extract

Graph 5a: is an HPLC of a countercurrent purified mycolic

acids from crude extract of *M. tuberculosis,* not extracted with acetone

Graph 5b: is an HPLC of a countercurrent purified reagents from crude reagent extract, not extracted with acetone

Graph 5c: is an HPLC of a countercurrent purified medium from crude medium extract, not extracted with acetone

Graph 6a: is an HPLC of an acetone supernatant of mycolic acids from *M. tuberculosis,* extracted with acetone after countercurrent purification

Graph 6b: is an HPLC of an acetone supernatant of reagents extracted with acetone after countercurrent purification

Graph 6c: is an HPLC of an acetone supernatant of medium extracted with acetone after countercurrent purification

Graph 7a: is an HPLC of purified mycolic acids from *M. tuberculosis,* extracted with acetone after countercurrent purification

Graph 7b: is an HPLC of purified reagents, extracted with acetone after countercurrent purification

Graph 7c: is an HPLC of a purified medium, extracted with acetone after countercurrent purification

Graph 8a: is an HPLC of a crude extract from *M. vaccae*, resaponified

Graph 8b: is an HPLC of mycolic acids from *M. vaccae*, rinsed with acetone, after countercurrent purification.

A schematic representation of the various purification procedures performed on crude extracts of bacterial cell-wall components and the various stages at which HPLC/s were performed is set out in Figure 3 below:

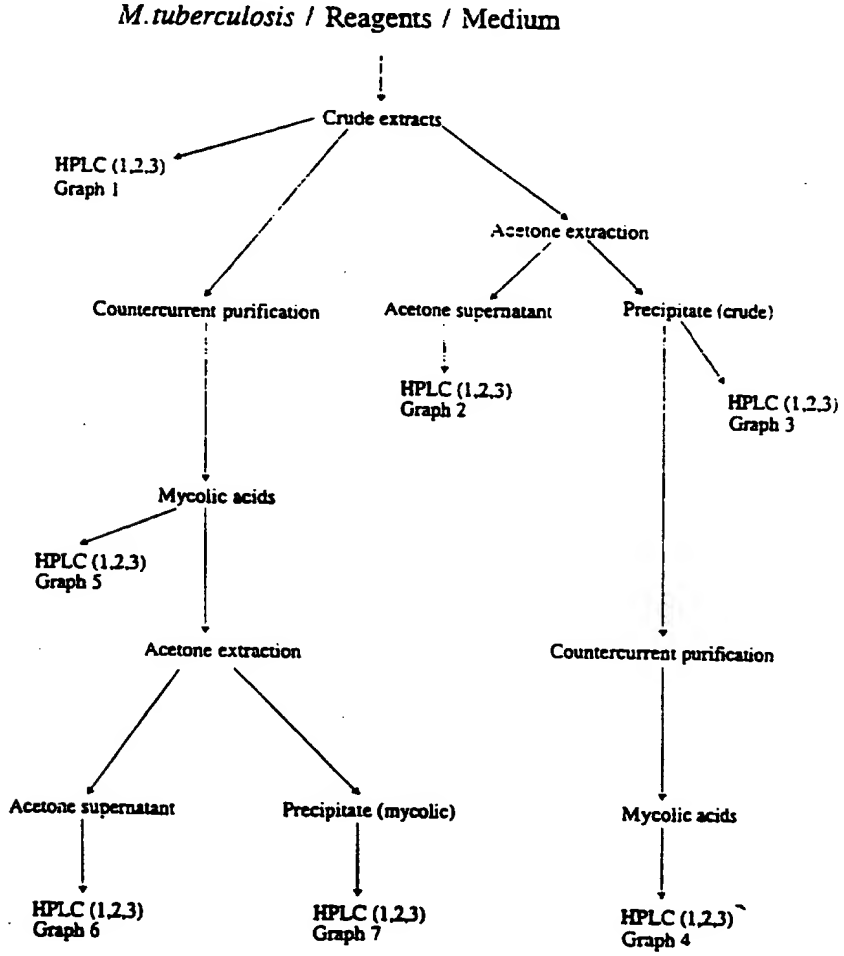


Fig. 3 Diagram of purification of mycolic acids from the crude lipid cell-wall extract originating from *M. tuberculosis*

DESCRIPTION OF PREFERRED EMBODIMENTS

The Aim of the Invention

The aim of the present invention is to isolate and purify microbial lipid cell-wall components and derivatives and analogues thereof, particularly mycolic acids originating from various microbial strains, to be used in:

1. The development of anti-tuberculosis and other anti-mycobacterial immunoprophylactic preparations, such as vaccines, for human and veterinary use. Such preparations can be based on mycolic acids with or without the concomitant presence of other mycobacterial cellular components and may or may not require the use of adjuvants of various types, including interleukines. The development of such preparations will include any manipulation necessary to render mycolic acids or other cell-wall lipid components immunogenic (e.g. the preparation of immunogenic conjugates, subunit vaccines).

2. The development and production of diagnostic tests for the confirmation of the presence of mycobacterial cells in samples such as sputum, cerebrospinal fluid, blood, urine, stools etc.

3. The production and commercialisation of individual purified cell-wall components, for research or other purposes (*e.g.* to be used as standards or for the development of diagnostic tests).

4. The immunotherapy of illnesses of mycobacterial and other origin, including autoimmune side effects.

5. The immunotherapy of multi-drug resistant mycobacterial infections.

6. The development of a test (*in vitro* or *in vivo*) for determining the reaction of the humoral or cellular immune system (the degree of stimulation of humoral or cellular immune system) to either the immunization or infection process.

7. The development of novel anti-tuberculosis drugs, aimed at the inhibition of the cell-wall synthesis.

8. The immune control of neoplastic disorders.

A high degree of purity of mycolic acids is important for the development of immunoprophylactic preparations, such as vaccines, immunotherapy and/or for detection methods, and more specifically for:

i) the determination of the solvent system in which the preparation of conjugates is to take place;

ii) the preparation of the conjugates necessary for the immunization of the experimental animals;

iii) the determination of the efficiency of coupling of mycolic acids to carrier molecules, *i.e.* for the monitoring of the conjugation process;

iv) the preparation of other conjugates required for monitoring of the process of immunization and for

the development of an ELISA type immunoassay;

v) the assessment of the specificity of the produced antibodies and for their characterization;

vi) the development of novel anti-tuberculosis drugs, aimed at the inhibition of the bacterial cell-wall synthesis.

The present invention provides a method, using a bi-phasic solvent system for the simultaneous purification and separation of different classes of compounds, which may be cell-wall components or derivatives or analogues thereof which may be extracted from a culture of the relevant cells or which may be synthesized. In a preferred embodiment of the invention lipid cell-wall components of microbial origin can be separated from contaminating material and separated as a group from contaminants. Purified lipid cell-wall components, *e.g.* mycolic acids, as well as other lipid cell-wall components originating from bacterial cell walls of microorganisms such as *Mycobacteria, Corynebacteria, Nocardia* and *Rhodococci*, can be separated as a group by this method. Although the method is applicable to all *Mycobacteria, Corynebacteria, Nocardia* and *Rhodococcus* strains, the examples present the experimental details concerning two *Mycobacteria* strains.

In essence the method involves subjecting a crude extract of microbial cell-wall components, dissolved in a unique bi-phasic solvent system, to a countercurrent liquid/liquid separation to purify a specific cell-wall component on a large scale. In the case of mycolic acids, the method yields in the order of 3 to 12% of the dry weight of the crude cellular extract, of

purified mycolic acid.

The method involves harvesting a growth of bacterial, e.g. mycobacterial, cells followed by saponification and extraction of lipid cell-wall components. The saponification step is necessary to release the lipid component from the rest of the cell skeleton or to release the free fatty acid salt from any ester form thereof, and the extraction step is necessary to remove the saponification agents from the cell wall component/reagent mixture.

The crude cellular extract obtained from the large-scale extraction process is dissolved in the lower phase of the bi-phasic solvent system and an amount of the upper phase of the bi-phasic solvent system is added. This solution is then subjected to countercurrent distribution through a series of approximately 25 cycles, each cycle comprises a mixing of upper and lower phases, a separation of the phases and a transfer of the separated phases to clean upper and lower phases.

Methyl esterification of the purified lipid cell-wall components occurs when they remain in methanol-containing solvents for extended periods. This is readily reversed by saponification, i.e. adding Reagent A at room temperature and re-extracting as described in the Methods.

The various groups of fatty acids from the cell-wall can be identified in the various tubes by their emulsification patterns in the lower or upper phase. The mycolic acids fraction, for example, was easily recognised by its emulsification pattern mainly in the lower phase within the first few tubes. The countercurrent-separated material is then withdrawn from the tubes. The mycolic acids fraction, even after being purified by countercurrent

distribution, was still found to contain some contaminating material. An amount of acetone was added to the purified sample and this was found to extract these impurities. (It was found that if acetone was added to the crude extract, some but not all of the impurities, which could stem from the ingredients of the bacterial culture medium, were extracted.) The purified mycolic acids were subjected to HPLC analysis to determine their purity, profile and yield.

The method of the invention was applied to the purification of mycolic acids from *M. tuberculosis*, *M.avium* and *M.vaccae* and proved to be effective for all of these strains of mycobacteria. Thus, using the method of the invention, it has been possible for the first time to separate a relatively large quantity of mycolic acids or other specific lipid cell-wall components and to purify them for subsequent use, such as in any one of the applications listed above.

EXAMPLES

MATERIALS

Cultures

Mycobacterium tuberculosis H37Rv ATCC 27294 - a virulent strain, originally isolated from an infected human lung, was used in the experiments.

Mycobacterium vaccae ATCC 15483 - a strain originally isolated from cow's milk.

The cultures were purchased in lyophilized form from the American Type Culture Collection (ATCC), Maryland, USA.

Media

The following media were used for the cultivation of *M. tuberculosis* and *M. vaccae*:

Liquid medium:	Dubos broth
Solid media:	Löwenstein-Jensen (LJ) medium
	Middlebrook 7H-10 medium

The media were prepared as recommended by Kent and Kubica (1985) in a Guide for the Level III Laboratory, Public Health U.S. Department of Health and Human Services, Atlanta.

Reagents

The following reagents were used in the purification of the extracted mycolic acids:

Chloroform (Saarchem, Analytical grade)

Methanol (Merck, Chemically pure)

Acetone (BDH, Analytical grade).

For the preparation of the reagents used for the extraction, derivatization and High-Performance Liquid Chromatography (HPLC) analysis of mycolic acids, HPLC Grade methanol and double-distilled deionized water were used.

Reagent A: 25% potassium hydroxide (Analytical Grade) dissolved in methanol-water (1:1): 62,5 g potassium hydroxide was dissolved in 125 ml water and 125 ml methanol (BDH, HPLC Grade) was added.

Reagent B: Concentrated hydrochloric acid (BDH, Analytical Grade) diluted 1:1 with water.

Reagent C: 2% potassium bicarbonate (BDH, Analytical Grade) dissolved in methanol-water (1:1): 10 g potassium bicarbonate was dissolved in 250 ml water and 250 ml methanol was added.

Reagent D: para-bromophenacylbromide in Crown Ether (Pierce Chemical Co, Cat. No 48891) was dispensed in 500 µl quantities into small amber-coloured screw cap vials with Teflon-coated septa. The caps were tightened and the vials were wrapped with Parafilm. Reagent D was stored at 4°C.

Reagent E: Reagent E was prepared by mixing reagent B 1:1 with methanol.

HPLC Standard:

High Molecular Weight Internal Standard (C-100) from Ribi ImmunoChem Research Company, Cat No R-50. The standard, 1 mg, was suspended in 2,0 ml methylene chloride (BDH, HPLC Grade), aliquots of 350 µl were dispensed into small screw-cap vials with Teflon-coated septa. The vials

were wrapped with Parafilm and stored at 4°C.

Chloroform (Associated Chemical Enterprises, Chemically Pure Grade)

Methylene chloride (BDH, HPLC-Grade)

Reagents A,B, C and E were prepared fresh prior to experiments, taking all the necessary safety precautions.

Countercurrent distribution apparatus

A countercurrent apparatus produced by H O POST, Instrument Company Inc., Middle Village, New York was used during the investigations. A photograph of the apparatus available at the Department of Biochemistry at the University of Pretoria, is presented in Figure 4. The "trains" in this model consisted of 2 X 250 inter-connected tubes.

Any other chemically-engineered system of countercurrent distribution may, of course, be used.

Infra-red spectrometer

Infra-red spectrum analyses of countercurrent-purified mycolic acids were carried out using a BOMEM Michelson 100 FTIR apparatus and a Hewlett Packard plotter.

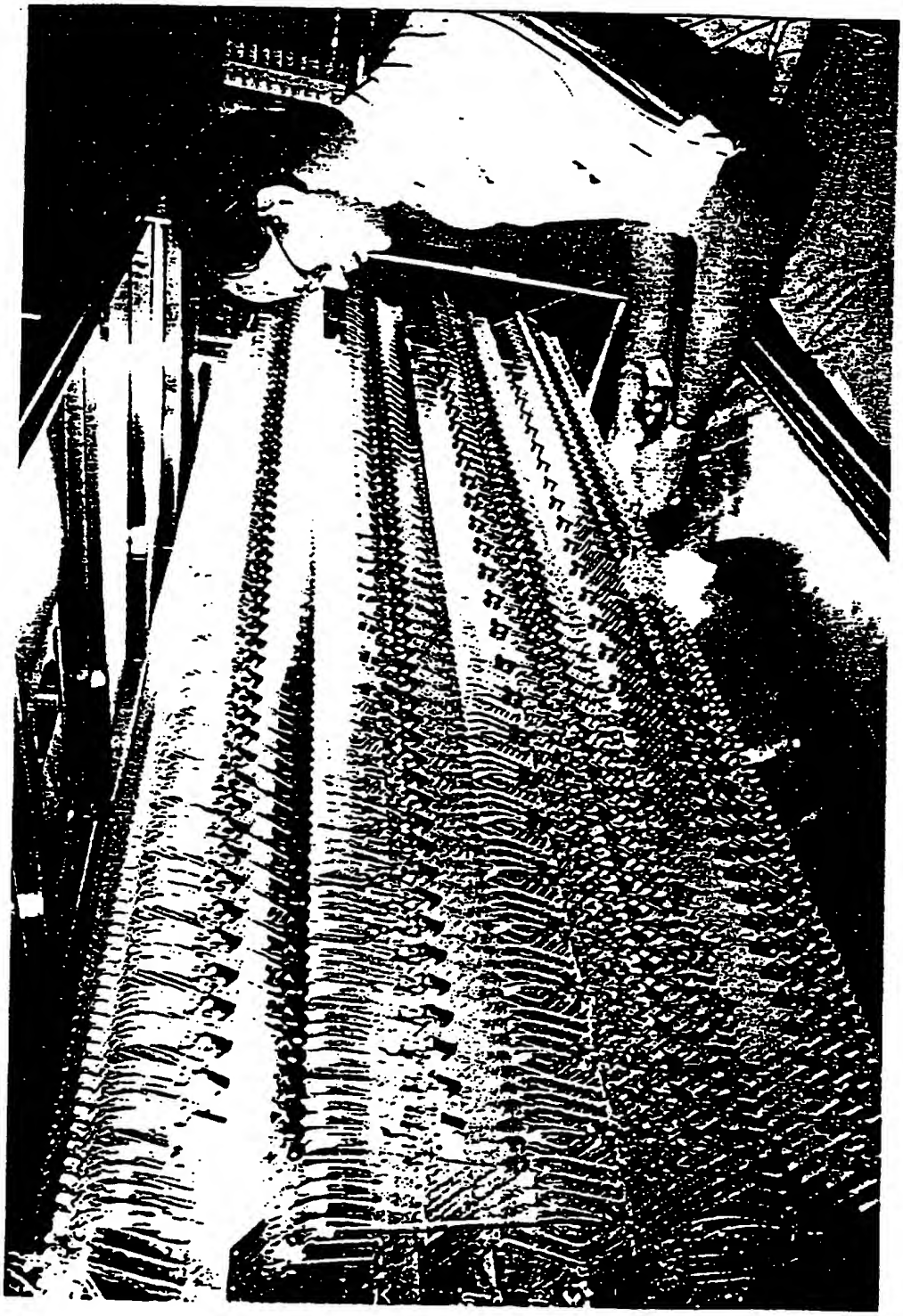


Figure 4. The countercurrent distribution apparatus used for the purification of mycolic acids.

METHODS

The following methods were used in the experimental work:

Cultivation of the bacterial strain

The bacteria were cultivated at 37°C using:

Dubos broth

Löwenstein-Jensen medium (slants), and

Middlebrook 7H-10 agar medium (slants).

The inoculation of the media and the handling of the *Mycobacterium* cultures, were carried out according to the procedures recommended by Kent and Kubica (1985) in a Guide for the Level III Laboratory, Public Health U.S. Department of Health and Human Services, Atlanta.

Preparation of lipid cell-wall components from bacterial samples

The preparation of bacterial samples comprised three steps:

harvesting of the *Mycobacteria* cells;

saponification and

extraction of fatty acids.

Harvesting was done by scraping the bacterial growth from the surface of media slants. The growth can also be collected from liquid cultures by centrifugation. Homogenous bacterial suspensions were prepared in Reagent A, by shaking or vortexing the harvested cells with sterile glass beads, to a final concentration of approximately 1 x 10^7 cfu*/ml.

Saponification of the *Mycobacteria* in Reagent A was carried out in an autoclave at 121°C, for one hour.

The **saponification, extraction and derivatization** of fatty acids were carried out as described by Butler, Jost and Kilburn (1991).

Extraction of lipid cell-wall components

The samples were allowed to cool and 1,5 ml Reagent B was introduced to each sample. After vortexing, the pH of each sample was checked and if necessary, adjusted to pH 1 with reagent B.

Subsequently, 2,0 ml chloroform was added to each sample and vortexed for 30 seconds. The layers were allowed to separate. The bottom layers were removed with Pasteur pipettes, transferred to WISP vials and evaporated to dryness at 85°C in a heat block-evaporator under a stream of nitrogen. To neutralize traces of acid carried over, 100 μl of reagent C was added to each sample and the fluid evaporated to dryness at 85°C on a heat block-evaporator under a stream of nitrogen. The samples were stored in the dark, under acetone at 4°C until used or analyzed by HPLC.

*cfu = colony forming units

Derivatization of fatty acids for HPLC analysis

Extracted and purified fatty acids (mycolic acids) were derivatized as follows:

Acetone was removed form the samples by evaporation on a heat block-evaporator at 85°C, under a stream of nitrogen. To each cooled sample 2,0 ml Reagent A was added. The samples were vortexed for 30 seconds, Reagent B (1,5 ml) was introduced, the samples were mixed again and the pH adjusted to pH 1,0, if required. Subsequently, chloroform (2,0 ml) was introduced, followed by the addition of 100 µl of Reagent C. The capped samples were vortexed for 30 seconds, heated for 5 minutes at 85°C on a heat block-evaporator and dried by introducing a stream of nitrogen.

Derivatization was carried out by introducing 1,0 ml chloroform and 100-150 µl of Reagent D, vortexing the samples for 30 seconds, sealing the vials containing the samples and placing them on a heat block-evaporator at 85°C for 20 minutes. Reagent E (1,0 ml) was added after the samples were cooled. The samples were vortexed again for 30 seconds and the layers allowed to separate. The bottom layers were removed with Pasteur pipettes and transferred to WISP-vials. The vials were placed on a heat block-evaporator and their contents evaporated to dryness at 85°C using a stream of nitrogen.

The residues were resuspended in 0,212 g (which corresponds to 160 µl) methylene chloride, capped and vortexed. Each reconstituted sample, into which 5 µl HPLC internal standard was introduced, was filtered through a 0,45 µm membrane filter into an amber-coloured WISP-vial. The recapped vials were stored at 4°C until ready for HPLC analysis.

HPLC analysis and quantification of mycolic acids

For the HPLC analysis 25 μl from each sample (maintained on ice during handling), was analyzed. Control samples, *i.e.* 25 μl of filtered methylene chloride, were run prior to each set of samples analyzed. If a large number of samples was analyzed, in order to validate the reliability of the HPLC apparatus, control samples were run after every three or four test samples.

The reverse-phase HPLC analyses were carried out using a Waters System High Performance Liquid Chromatography apparatus (Milford, MA) consisting of:

System Controller (Waters 600 E);

Detector (Waters 486 Tunalde);

Autosampler (Waters 712 WISP);

Data module (Microsep M 741);

Column Waters (Nova-Pak C18 60 A, 4 μm, 3,9 x 150 mm) and an end connector set for steel cartridge columns;

Guard column (Guard-Pak/Nova-Pak C18).

Running conditions were:

Mobile phase:

Solvent A: HPLC Grade Methanol

Solvent B: HPLC Grade Methylene chloride

Flow Rate: 2,5 ml/min

Column temperature: 30°C

The detector was set at 260 nm.

The HPLC gradient initially comprised 98% (v/v) methanol (Solvent A) and 2% (v/v) methylene chloride (Solvent B). The gradient was increased linearly to 80% A and 20% B at one minute; 35% A and 65% B at ten minutes, held for 5 seconds and then decreased over 10 sec back to 98% A and 2% B. This ratio was maintained for 6 minutes to allow for stabilization of the system prior to injection of the next sample.

Mathematical quantification of mycolic acids was carried out by comparing the combined peak areas of the tested samples to the peak area of the introduced quantity of the High Molecular Weight Internal HPLC Standard.

Purification of mycolic acids and group separation of other fatty acids

A countercurrent distribution train comprising 25 tubes, numbered 0-24, was used in the experiment. Into a buffer reservoir approximately 900 ml of the upper phase was introduced.

Into tube number 0, a sample of the crude cellular extract of *M. tuberculosis* obtained from a large-scale extraction experiment (30 - 150 mg), dissolved in 10 ml of the lower phase and 10 ml of the upper phase was introduced. Into the remaining 24 tubes aliquots of 10 ml of the lower phase were introduced. Upper phase, in volumes of 10 ml per cycle, was automatically dispensed into tube number 0, repeatedly over 25 cycles resulting in approximately 16 hour operation. Thus, twenty five countercurrent cycles were performed, with each cycle consisting of 20 mixing pendula and 40 minutes phase separation time.

The process is graphically illustrated in Fig. 5.

With each transfer, any solute originating from the sample and present in the upper phase is carried into the succeeding tube. After the completion of twenty five transfers, the separated solute fractions should be distributed along the train of 25 tubes.

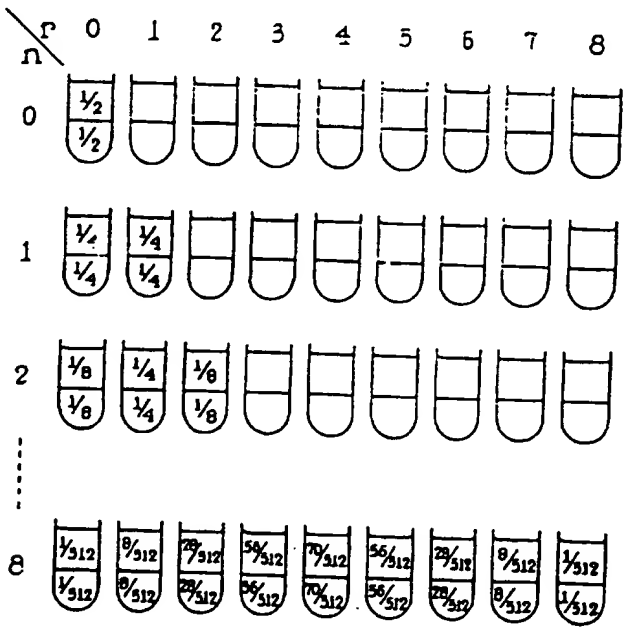


Fig. 5 A schematic representation of countercurrent distribution system with nine tubes.

To establish the distribution of fatty acids among the twenty five tubes, the emulsification patterns in upper and lower phases were observed within the tube train and fractions were determined accordingly.

The countercurrent-separated material was then withdrawn from the tubes using a 50 ml glass syringe, with a Teflon tubing attached. The material was pooled into seven fractions, dried individually under vacuum in a Buchi evaporator at 70°C, the dried material redissolved in either chloroform, methanol or water (in approximately 5 ml), transferred into amber WISP-vials and stored at 4°C until required.

Acetone extraction

In order to remove any residual impurities still present in the countercurrent-purified material, an additional extraction with acetone was carried out as follows:

> to a sample of the purified mycolic acids (approximately 5,0 mg) placed in a WISP vial, an aliquot of acetone (3,0 ml) was introduced and the sample was mixed by vortexing. After sedimentation, the acetone was removed by aspiration and the process repeated two times. The finally purified and extracted sample was stored under acetone.

Yield of the mycolic acids purified by countercurrent separation

In order to calculate the approximate yield of purification/separation, the weighed amount of the mycolic acids present in the samples obtained after the countercurrent separation/purification was compared to the amount of

these compounds present in the crude cellular extract introduced into the countercurrent apparatus, calculated from the relative peak areas of the mycolic acids peak cluster and that from the peak area of the standard in the HPLC chromatogram of the crude cellular extract.

Alternatively, the peak area from the mycolic acids peak cluster of the HPLC chromatogram of the purified mycolic acids was compared to that obtained for the crude extract to estimate the yield and purity of the product.

Infra-red spectrum analysis of the purified mycolic acids

Infra-red spectrum analysis was carried out using a countercurrent-purified sample (1 mg) extracted from *M. tuberculosis*. Mycolic acids were dissolved in 1 ml HPLC grade chloroform and injected into the liquid vial. As a reference HPLC grade chloroform was used. The absorption of the reference was subsequently subtracted from the sample profile.

RESULTS

Purification of mycolic acids from *M. tuberculosis*

The material (37,5 mg) extracted from *M. tuberculosis* H37Rv (ATCC 27294), according to the method proposed by Butler, Jost and Kilburn (1991) for the isolation of mycolic acids, was found to contain less than 10% mycolic acids after countercurrent extraction of 25 cycles (Fig. 6). Mycolic acids were identified by HPLC in fractions 1 and 2 only, which together comprised 9,3% of the dry mass of the crude extract that was loaded on the countercurrent apparatus. Mycolic acids exhibited a

distribution coefficient of only 0,08, which allowed complete separation from the shorter saponified fatty acids at a distribution coefficient of around 1,26 (Fig. 6). The mycolic acid fraction was easily recognised by its emulsification pattern in the countercurrent tube train in the first few tubes, followed by 3 tubes which did not contain any significant amount of material. The saponified short chain fatty acids could be identified in 4 tubes containing emulsion in the lower phase, immediately followed by 8 tubes with emulsion in the upper phase, indicative of the equilibrium between dissociated (upper phase emulsion) and protonated (lower phase emulsion) fatty acids during the separation. The remaining fractions 6 and 7 could not be analyzed by HPLC due to the fact that they were not soluble in chloroform, a property which also excludes the possibility of their containing any mycolic acids.

The HPLC profiles/chromatograms of the crude extracts originating from *M. tuberculosis*, the reagents used and the bacterial medium are presented in Graphs 1a, 1b and 1c, respectively.

Mycolic acids, purified by countercurrent distribution, still contained some contaminating material which could be detected on HPLC at a retention time spanning from 4-6 minutes (Graph 5a). A diagram showing how acetone was employed to extract these impurities from the samples, is given in Fig. 3. The diagram represents a systematic investigation on a single large batch of crude extract originating from *M. tuberculosis* to allow direct comparison of HPLC profiles and calculate relative yields.

The contaminating peaks did not arise from the reagents used in the extraction and purification procedures (Graph 5b), but could stem from the ingredients of the bacterial culture medium (Graph 5c). When extraction

with acetone was carried out on the crude extracts prior to countercurrent purification, some degree of extraction of impurities from bacterial (Graph 2a) and growth medium (Graph 2c) samples was observed, but it did not suffice to remove all impurities from the final countercurrent-purified product (Graph 4a). When the dried, countercurrent-purified mycolic acids were extracted with acetone, it appeared that the impurities were soluble in the acetone (Graphs 6a and 6c). The HPLC analysis of the acetone-rinsed mycolic acids fraction indicated that this fraction was free from any impurities (Graph 7a), other than those which were due to the derivatization reagents and which could be observed in control extracts from purification reagents (Graph 7b) and growth media (Graph 7c).

On the basis of these results it is claimed that a crude extract of *Mycobacteria*, obtained according to the method of Butler, Jost and Kilburn (1991) and purified by countercurrent separation as described above followed by acetone extraction, yields mycolic acids free from any impurities detectable by HPLC (Graph 7a).

The purity of mycolic acids was also assessed by comparing the mass of the countercurrent-purified material with the calculated yield of mycolic acids based on the surface of the absorbance peaks on the HPLC chromatogram. Countercurrent-purified mycolic acids before acetone rinsing (Graph 5a; 0,3 mg) were analyzed by HPLC after derivatization as described above. When the surface of the absorption peak of the internal standard (representing 5,01 μg) was compared to that of the mycolic acids, a yield of 0,27 mg of mycolic acids was calculated, representing 90% of the weighed material. Taking into consideration that some material is lost by transfer of phases between vials during the derivatization and extraction process, and that some contamination is removed by acetone extraction, the

purity of the mycolic acids fraction represented by the HPLC profile in Graph 7a may be regarded as approaching 100%.

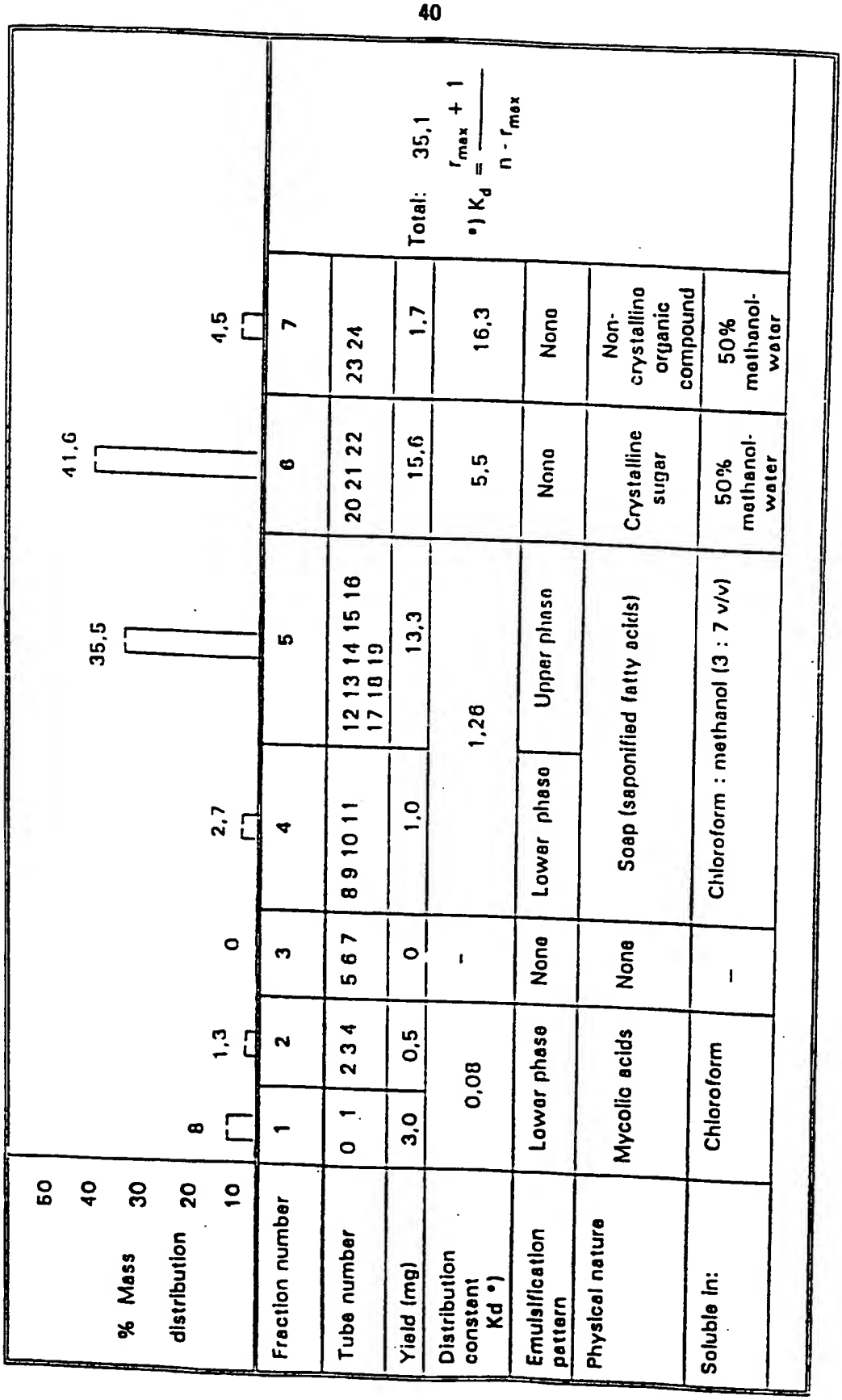
Countercurrent purification yielded mycolic acids as methyl ester derivatives. This is evident from the infrared spectrum of the countercurrent purified mycolic acids before acetone extraction (Fig. 7) and by HPLC analysis when resaponification in Reagent A after purification is omitted (results not included). The addition of Reagent A restores the free acid and is required to effect derivatization with para-bromophenacylbromide.

Purification of mycolic acids from *M. vaccae*

The growth of *M. vaccae* was extracted and purified in the same way as that of *M. tuberculosis*. HPLC analysis of the crude extract (Graph 8a) showed three peak clusters with retention times greater than 5 minutes and a broad reagents/short chain fatty acids peak cluster at a retention time shorter than 2 minutes. After countercurrent purification and rinsing with acetone, two clusters of peaks remained at retention times greater than 5 minutes on the HPLC column (Graph 8b). In addition, the reagents/short chain fatty acids peak cluster was considerably narrower, leaving no traces of detectable impurities. On a mass basis, the mycolic acids were purified to approximately 5% yield, whereas 4,3% yield was calculated by comparing the areas of the mycolic acids peak clusters of the HPLC chromatograms of the crude and purified mycolic acids extracts.

Similar results were obtained with countercurrent purified mycolic acids extracted from *M.avium* (ATCC 25291).

Besides somewhat lower yields, these results are similar to those found for the purification of mycolic acids from *M. tuberculosis*, thus demonstrating the wider application of the countercurrent purification method of mycolic acids among species of *Mycobacteria*.



Fraction number	1	2	3	4	5	6	7	
Tube number	0 1	2 3 4	5 6 7	8 9 10 11	12 13 14 15 16 17 18 19	20 21 22	23 24	Total: 35,1
Yield (mg)	3,0	0,5	0	1,0	13,3	15,6	1,7	
Distribution constant Kd *)	0,08		-	1,28		5,5	16,3	*) $K_d = \frac{r_{max} + 1}{n - r_{max}}$
Emulsification pattern	Lower phase		None	Lower phase	Upper phase	None	None	
Physical nature	Mycolic acids		None	Soap (saponified fatty acids)		Crystalline sugar	Non-crystalline organic compound	
Soluble in:	Chloroform		–	Chloroform : methanol (3 : 7 v/v)		50% methanol-water	50% methanol-water	

Fig.6 Countercurrent separation of components in the crude mycolic extract of *M.tuberculosis*

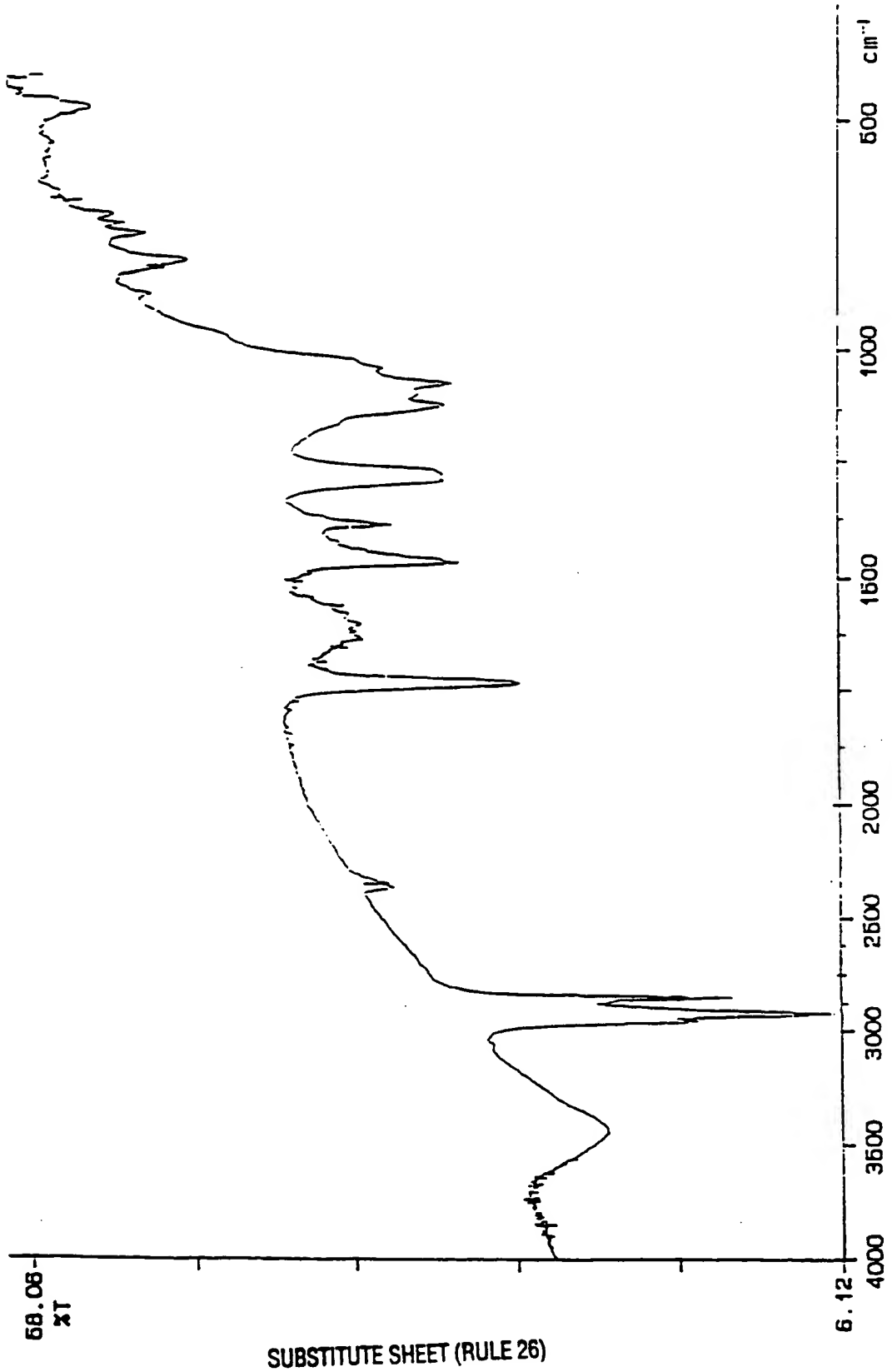


Fig. 7 Infra-red spectrum of the countercurrent-purified mycolic acids originating from *M.tuberculosis*

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CLAIMS

1. A method of separating and purifying a specific microbial cell-wall component or a derivative or analogue thereof from an extracted mixture of the cell-wall component or derivative or analogue thereof and contaminants or from a synthetic mixture of the cell-wall component or analogue or derivative thereof and contaminants comprising the steps of:

   dissolving the extracted mixture or synthetic mixture in a multi-phasic solvent to form a solution; and

   subjecting the solution to liquid-liquid phase extraction.

2. A method according to claim 1, wherein the specific cell-wall component or derivative or analogue thereof is a compound with immunoregulatory properties.

3. A method according to claim 1 or claim 2, wherein the specific cell-wall component or derivative or analogue thereof is a lipid or a sugar.

4. A method according to claim 3, wherein the specific cell-wall component is a lipid.

5. A method according to claim 4, wherein the lipid is a fatty acid.

6. A method according to claim 5, wherein the fatty acid is a mycolic acid.

7. A method according to claim 6 for separating and purifying a mixture of mycolic acids as a group from an extracted or synthetic mixture of mycolic acids and contaminants.

8. A method according to any one of claims 1 to 7, wherein the microbial cell-wall component is derived from a bacterium, a fungus or a yeast.

9. A method according to claim 8, wherein the microbial cell-wall component is derived from a bacterium.

10. A method according to claim 9, wherein the bacterium is selected from *Mycobacteria, Corynebacteria, Nocardia, Rhodococci* and *Amycolata*.

11. A method according to claim 10, wherein the bacterium is selected from *M tuberculosis, M.avium* and *M.vaccae*.

12. A method according to any one of claims 1 to 11, wherein the multi-phasic solvent system comprises chloroform, methanol and water.

13. A method according to claim 12, wherein the multi-phasic solvent system is a bi-phasic solvent system.

14. A method according to claim 13, wherein the bi-phasic solvent system comprises an upper liquid phase and a lower liquid phase.

15. A method according to claim 14, wherein the method also comprises the steps of mixing and equilibrating the upper and lower phases of

the solvent system.

16. A method according to claim 14 or claim 15, wherein the composition of the upper phase is 12-18% chloroform, 45-55% methanol and 25-40% water.

17. A method according to claim 16, wherein the composition of the upper phase is 15% chloroform, 52% methanol and 33% water.

18. A method according to any one of claims 14 to 17, wherein the composition of the lower phase is 50-80% chloroform, 15-40% methanol and 2-8% water.

19. A method according to claim 18, wherein the composition of the lower phase is 68% chloroform, 27% methanol and 5% water.

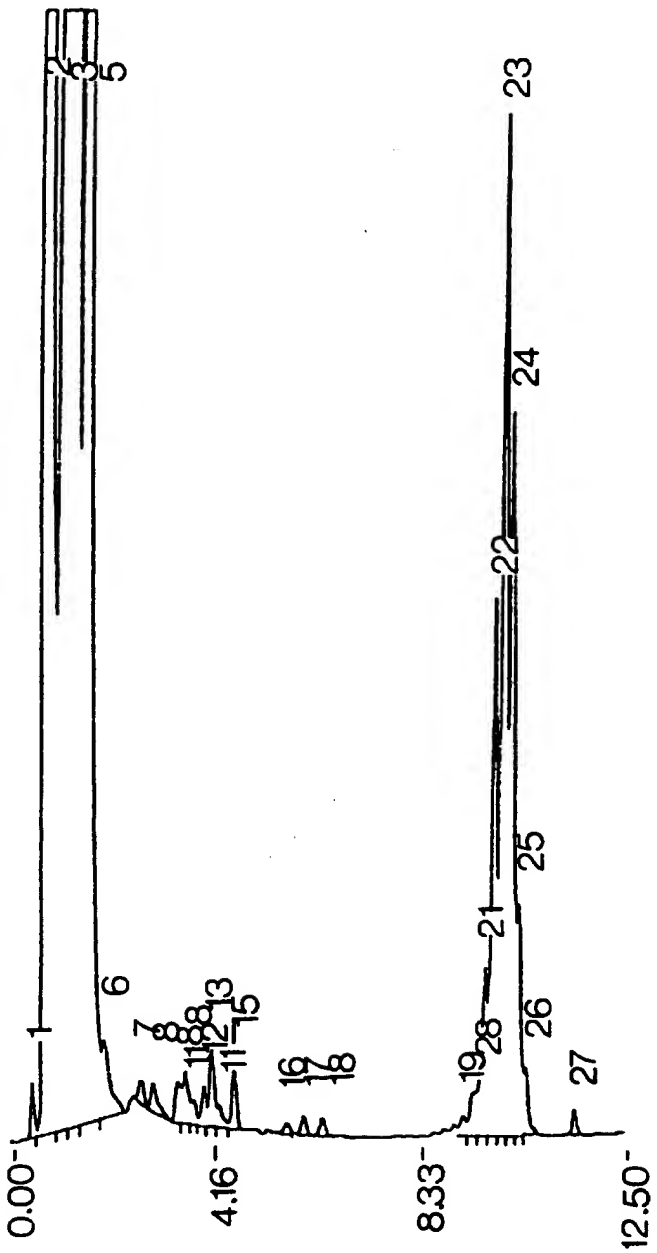
20. A method according to any one of the preceding claims, wherein the liquid-liquid phase extraction is a countercurrent extraction or a multiple extraction of one of the phases.

21. A method according to any one of the preceding claims, which also comprises the step of subjecting the purified cell-wall component or analogue or derivative thereof to an acetone extraction to remove impurities.

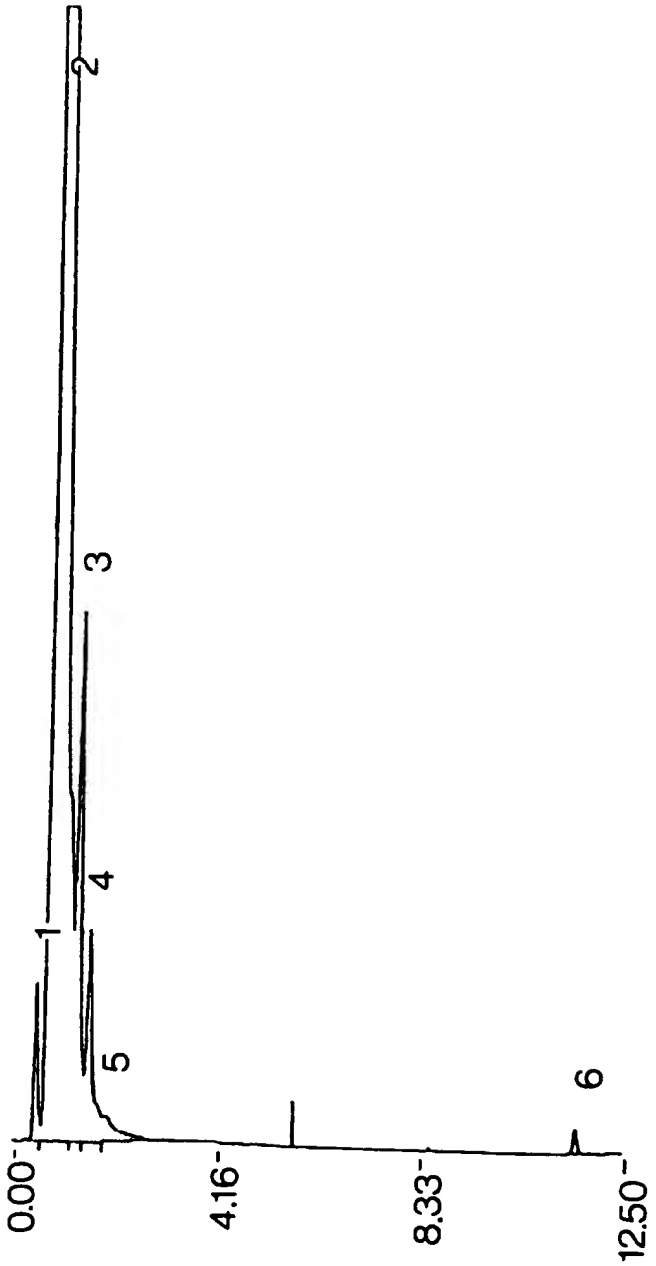
22. A method according to any one of the preceding claims, wherein the cell-wall component or analogue or derivative thereof does not require any chemical derivatisation to separate it from any impurities.

23. A method according to any one of the preceding claims which also comprises the step of saponifying the purified, separated cell-wall component or derivative or analogue thereof.

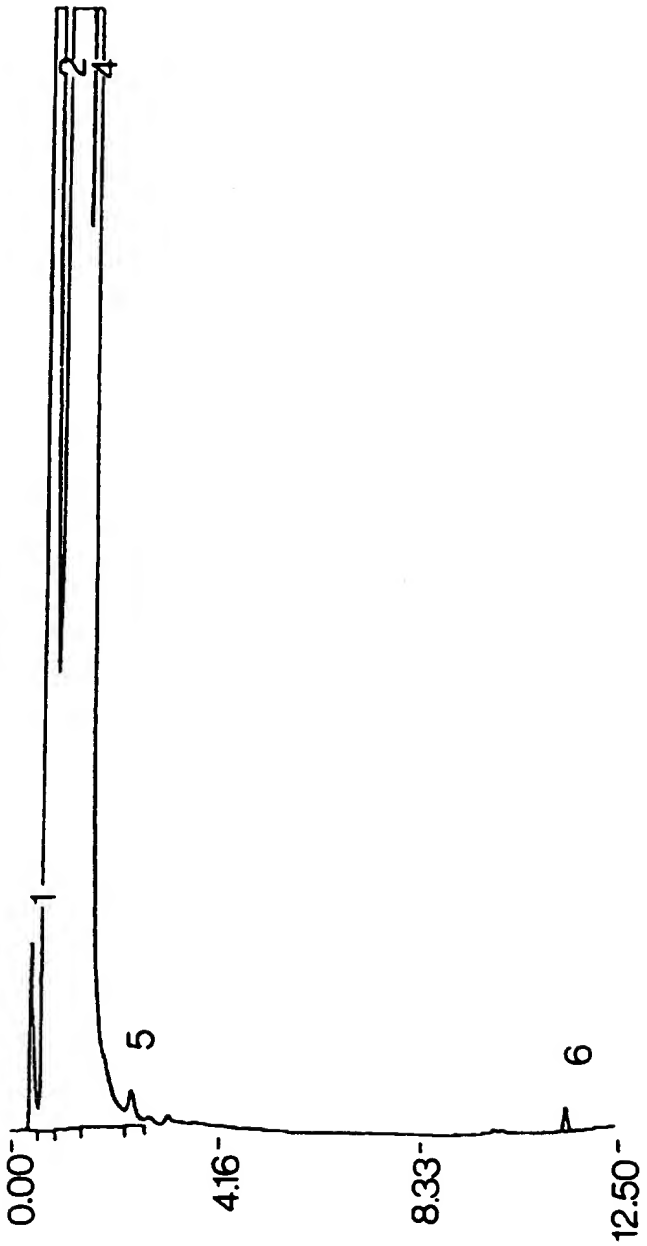
24. A method according to claim 1 substantially as herein described with reference to the illustrative examples.



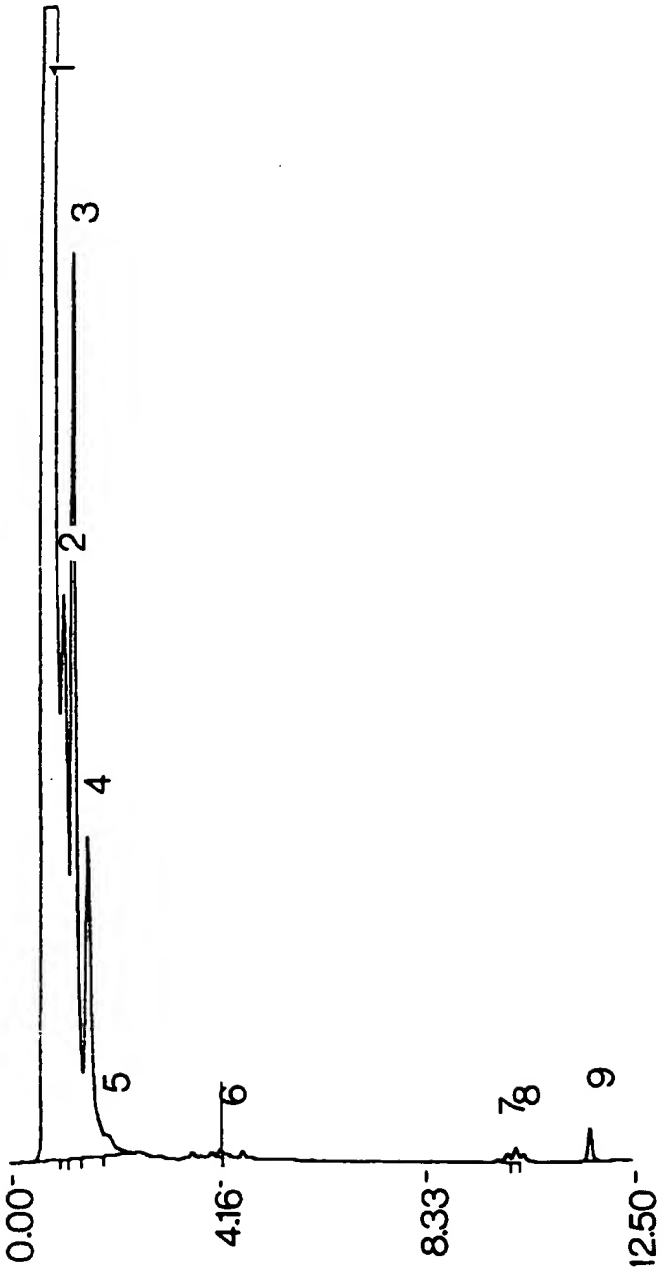
Graph 1a: Crude *M. tuberculosis* extract



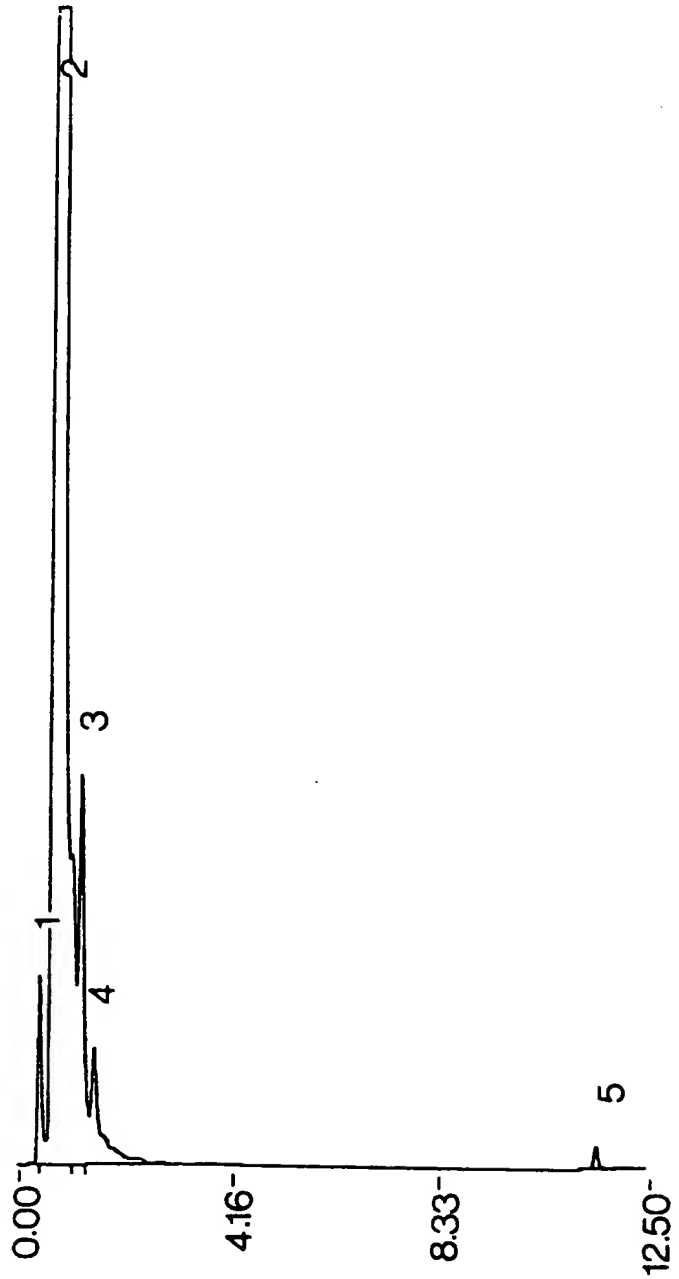
Graph 1b: Crude reagents extract



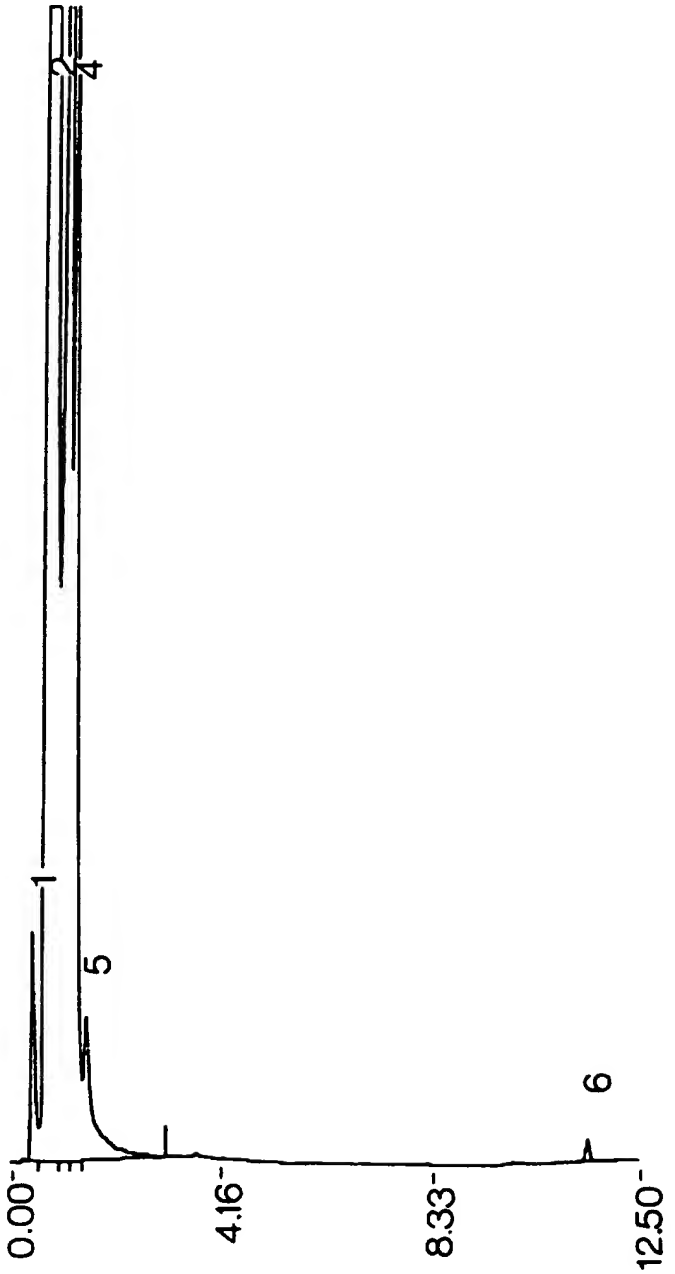
Graph 1c: Crude medium extract



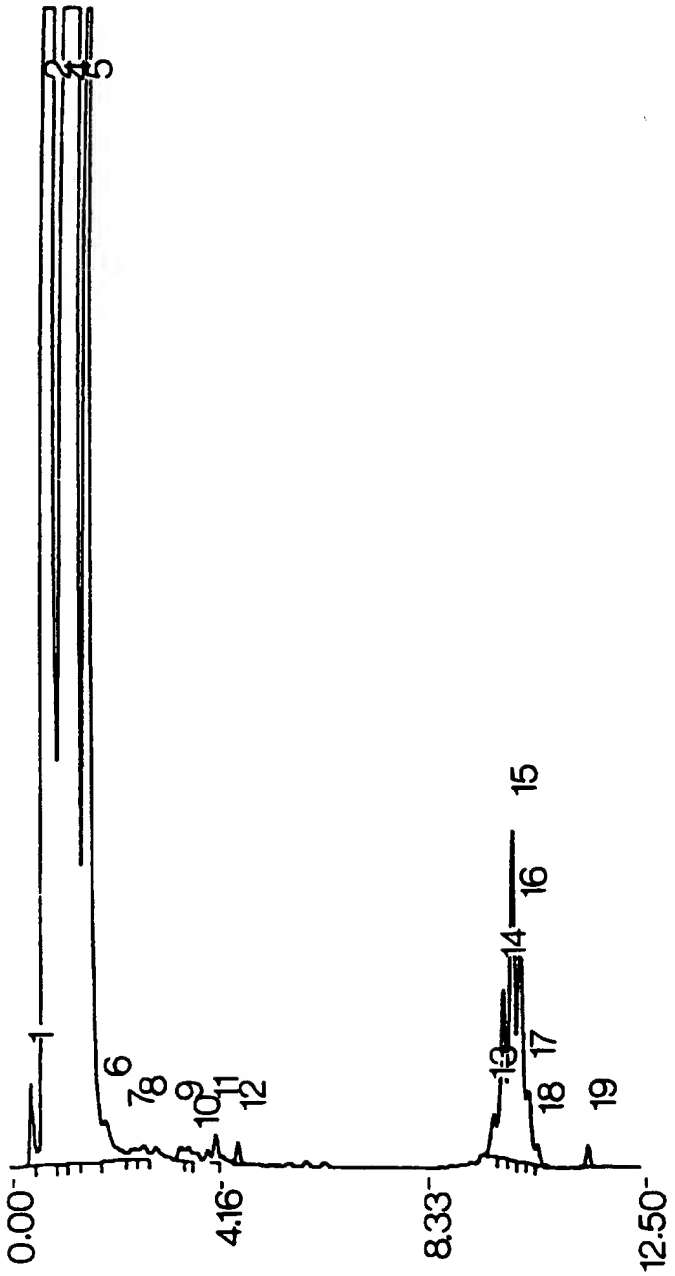
Graph 2a: Acetone supernatant of the extracted crude *M. tuberculosis* extract



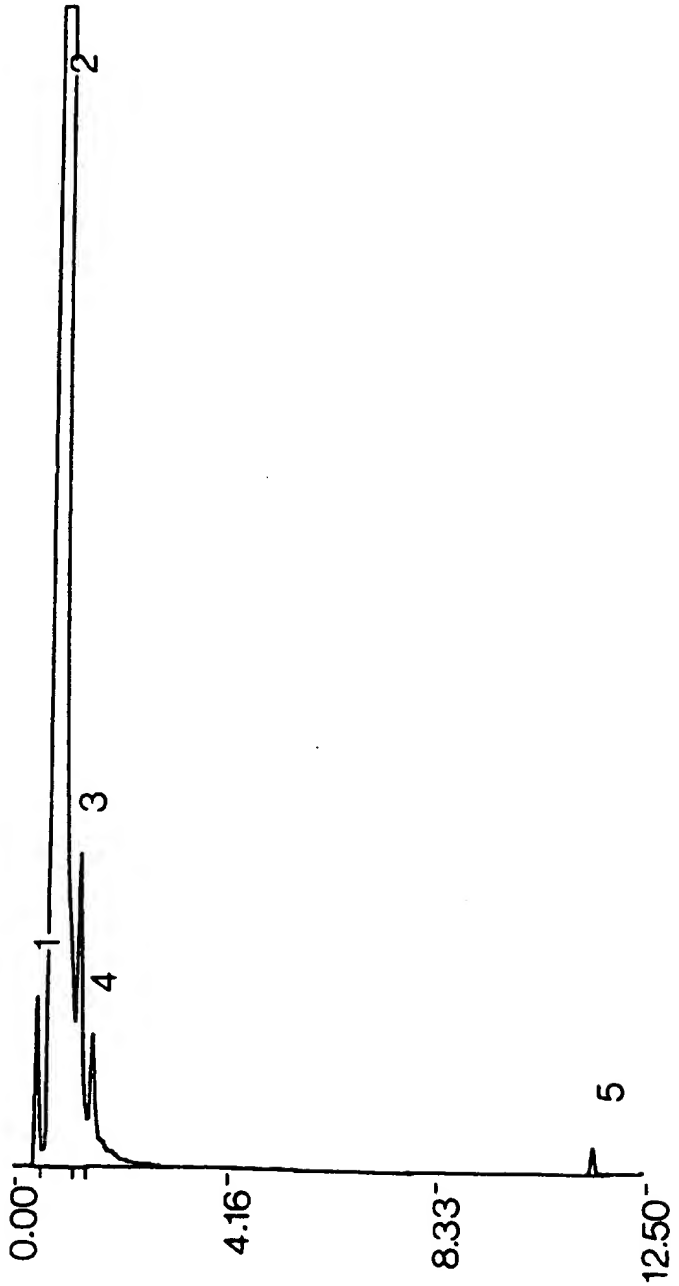
Graph 2b: Acetone supernatant of the extracted, crude reagents extract



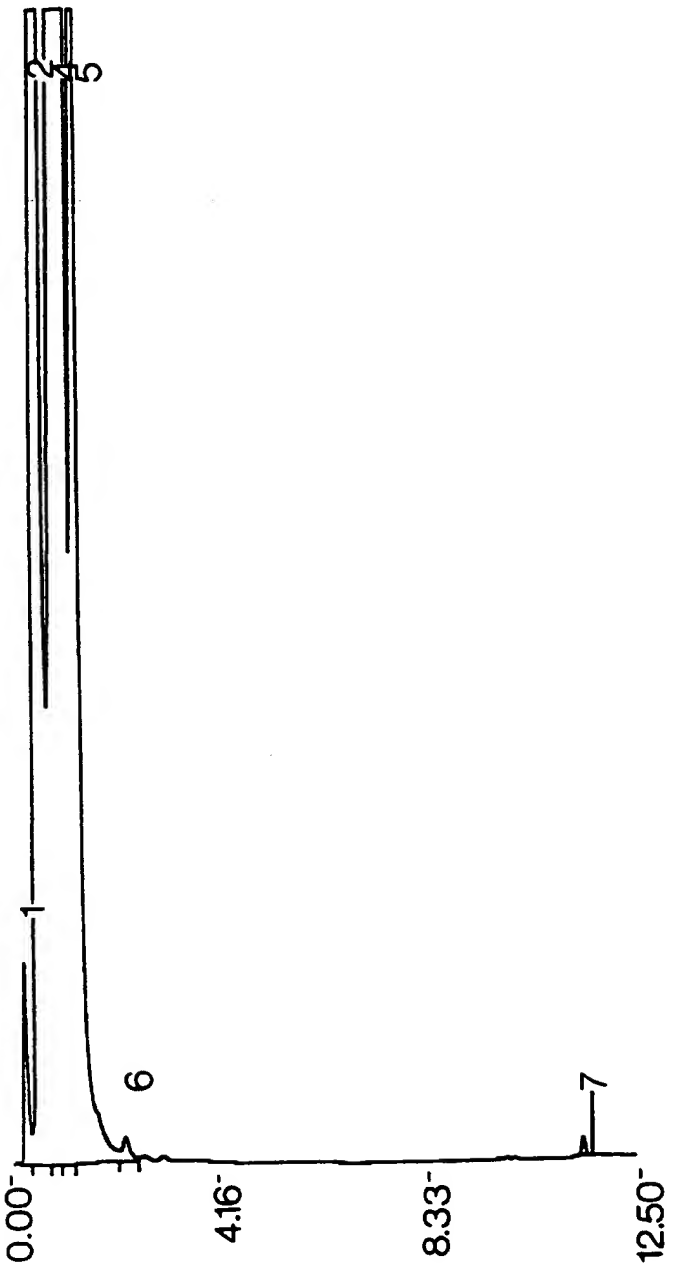
Graph 2c: Acetone supernatant of the extracted, crude medium extract



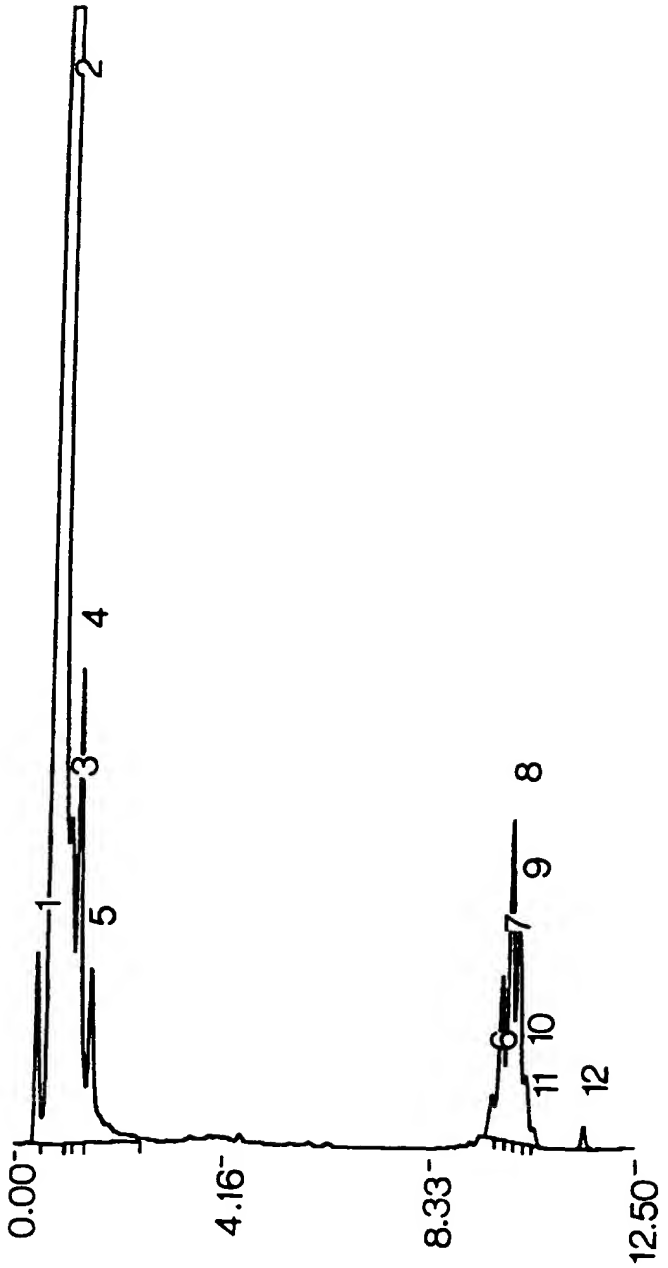
Graph 3a: Crude *M. tuberculosis* extract, acetone extracted



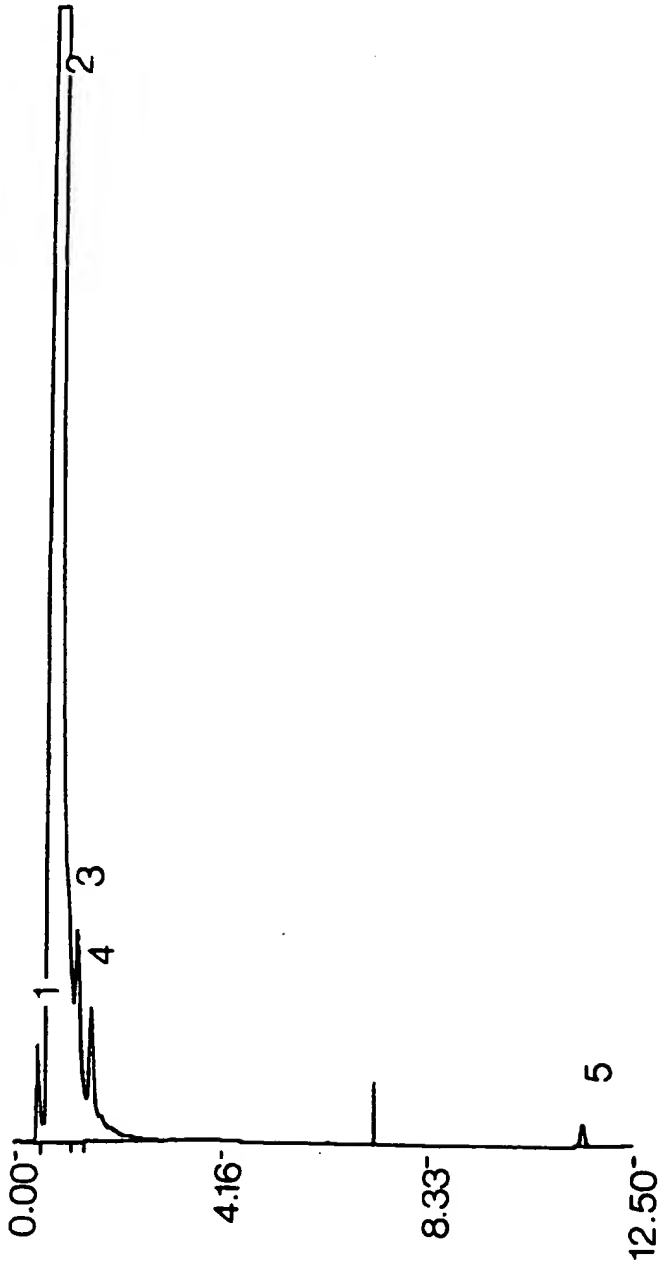
Graph 3b: Crude reagents extract, acetone extracted



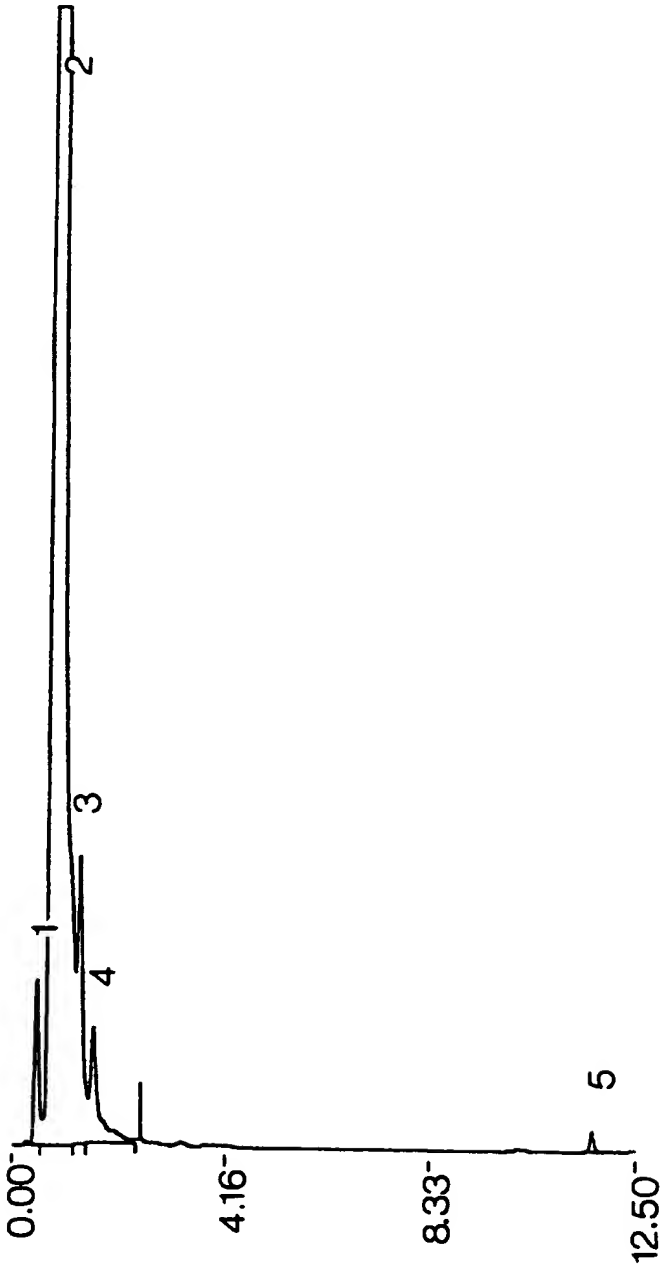
Graph 3c: Crude medium extract, acetone extracted



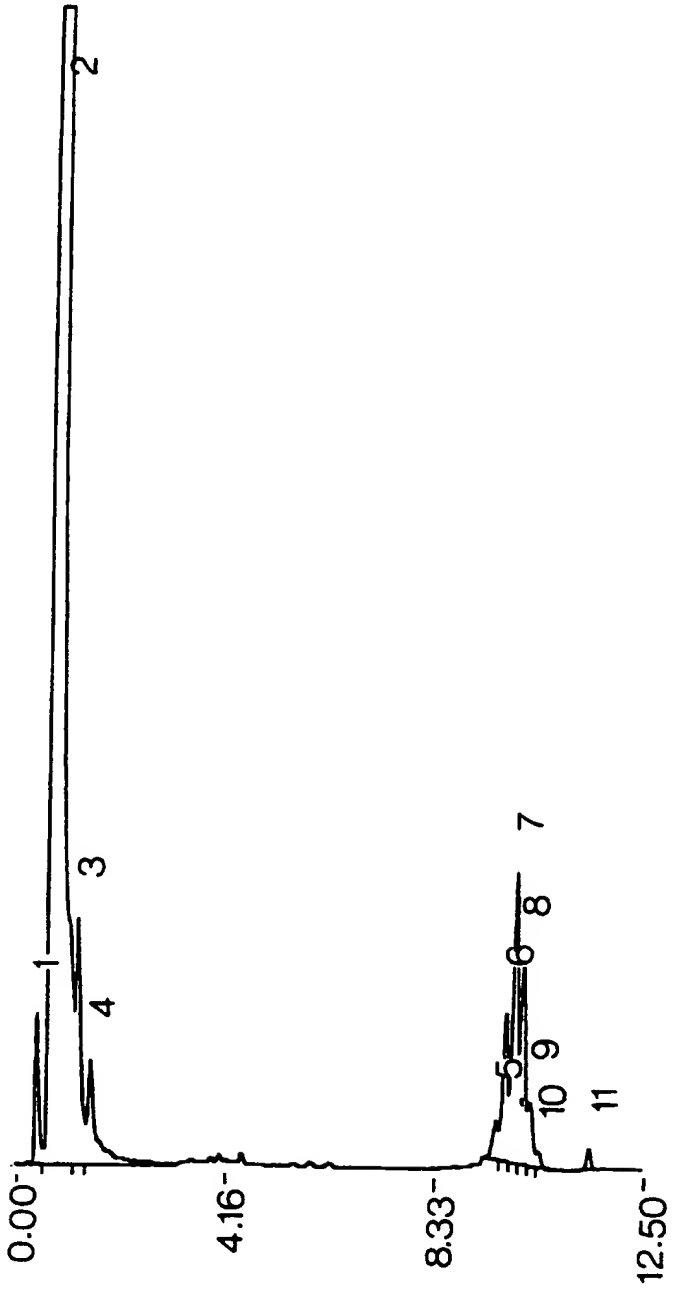
Graph 4a: Countercurrent purified mycolic acids of *M. tuberculosis*, from acetone extracted crude extract



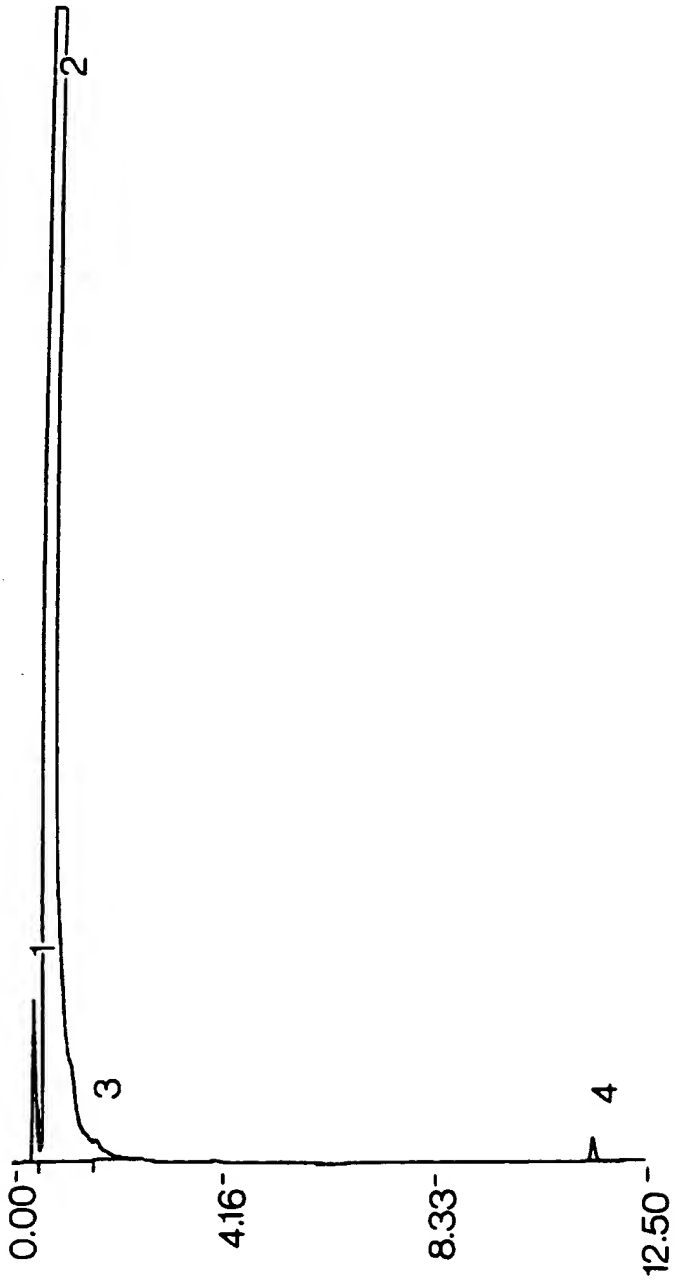
Graph 4b: Countercurrent purified reagents from acetone extracted crude reagents extract



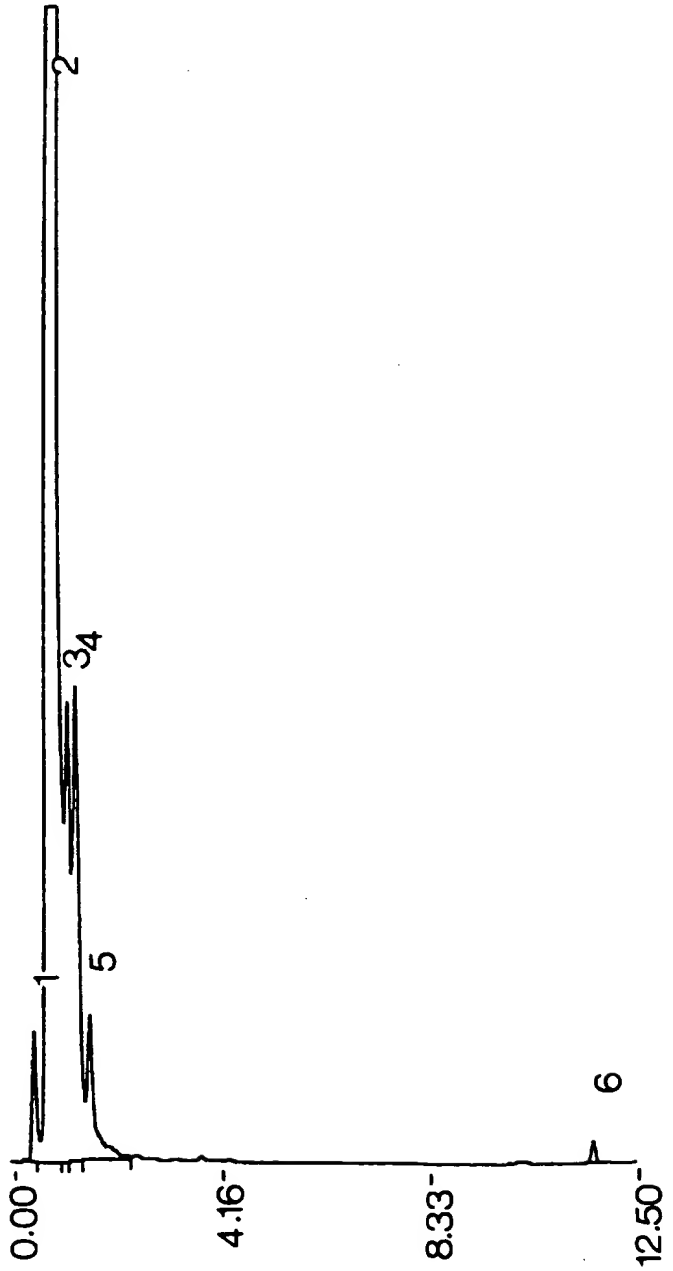
Graph 4c: Countercurrent purified medium from acetone extracted crude medium extract



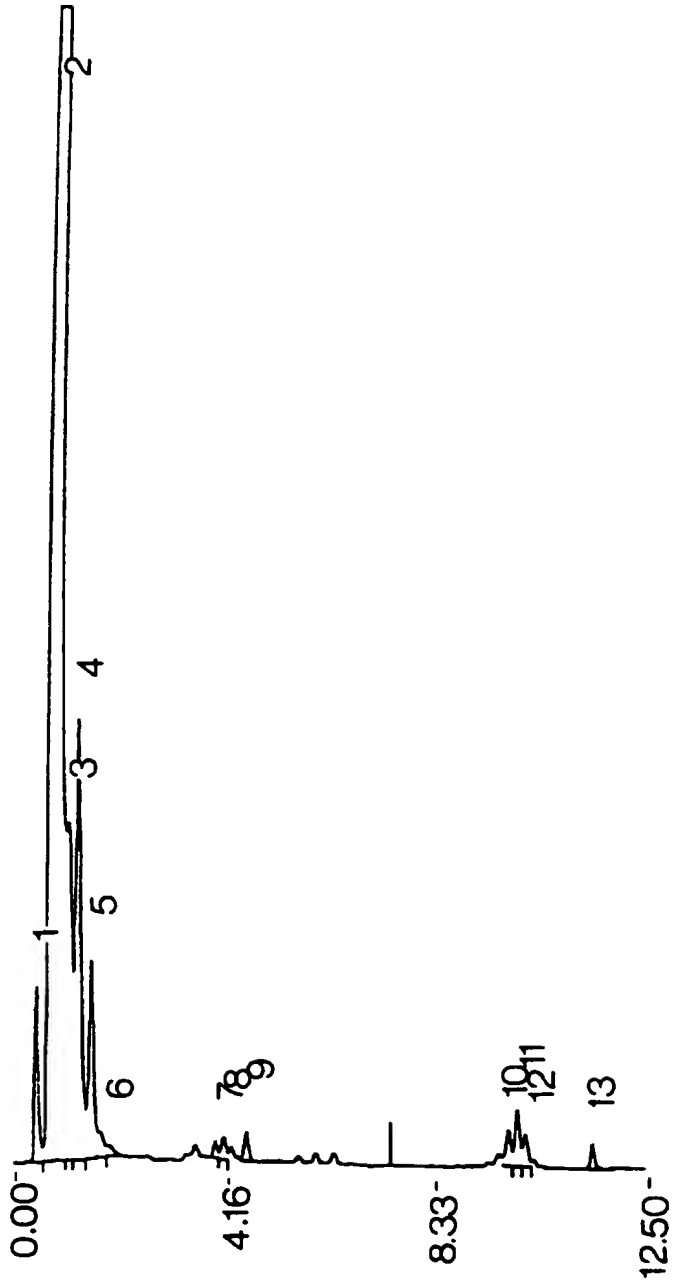
Graph 5a: Countercurrent purified mycolic acids from crude extract of *M. tuberculosis*, not extracted with acetone



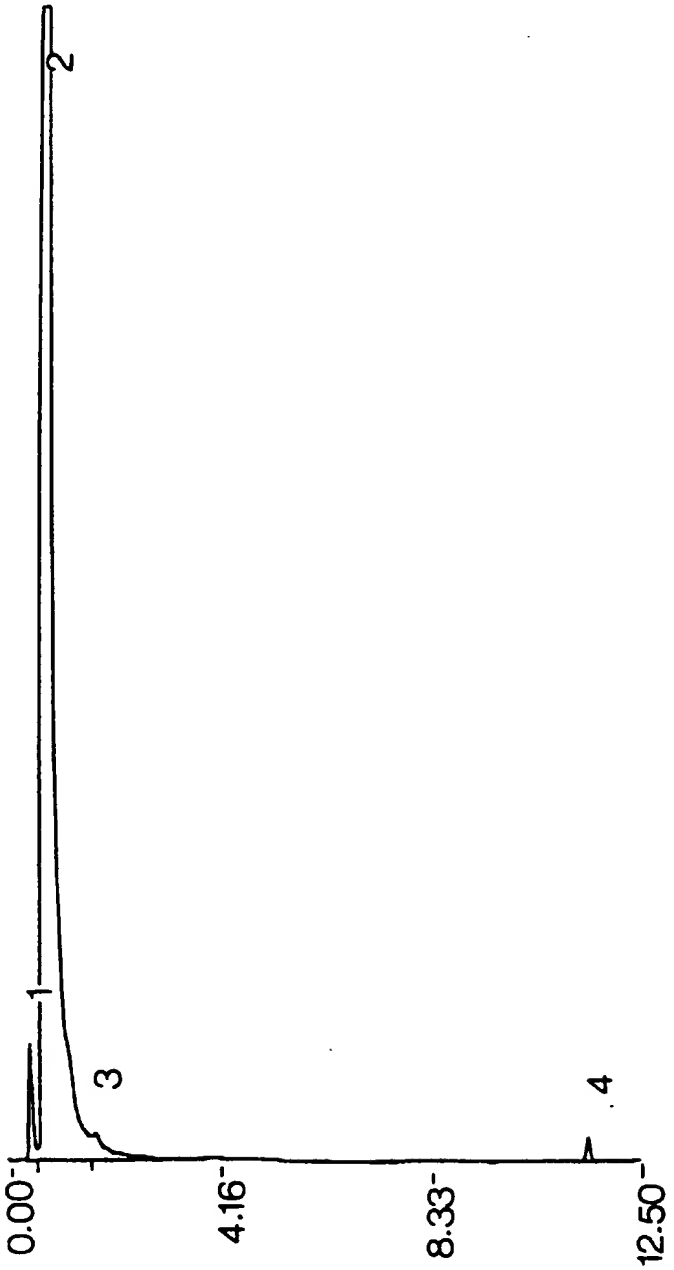
Graph 5b: Countercurrent purified reagents from crude reagent extract, not extracted with acetone



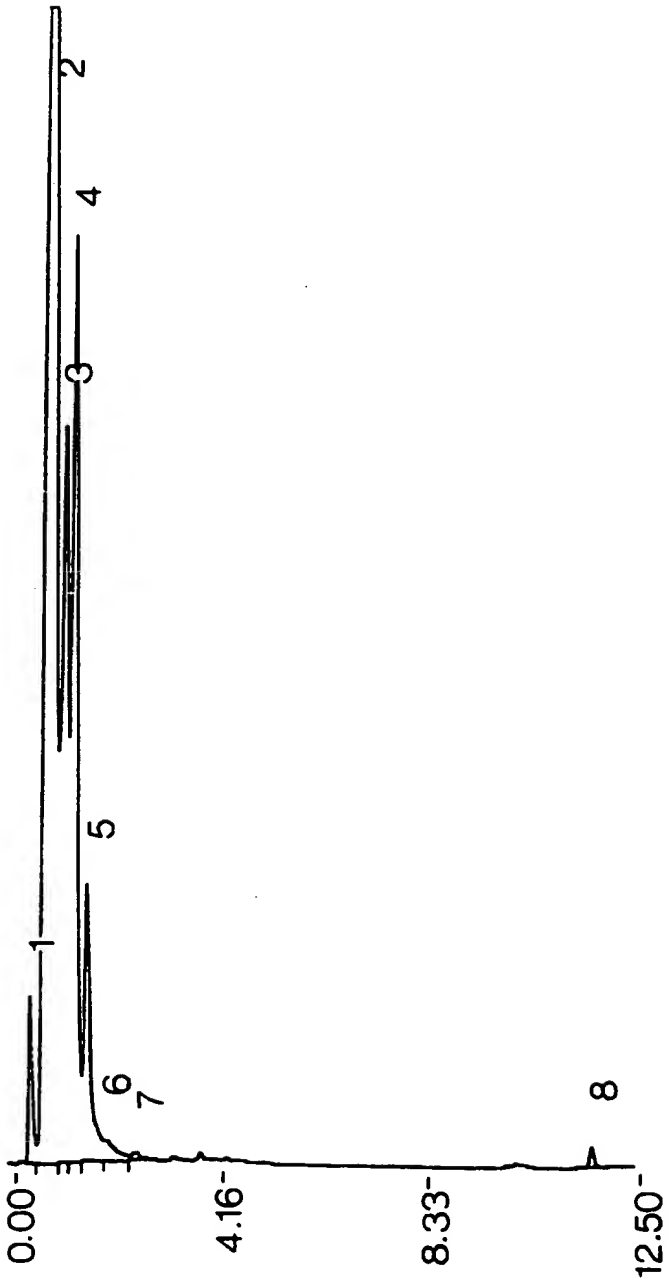
Graph 5c: Countercurrent purified medium from crude medium extract, not extracted with acetone



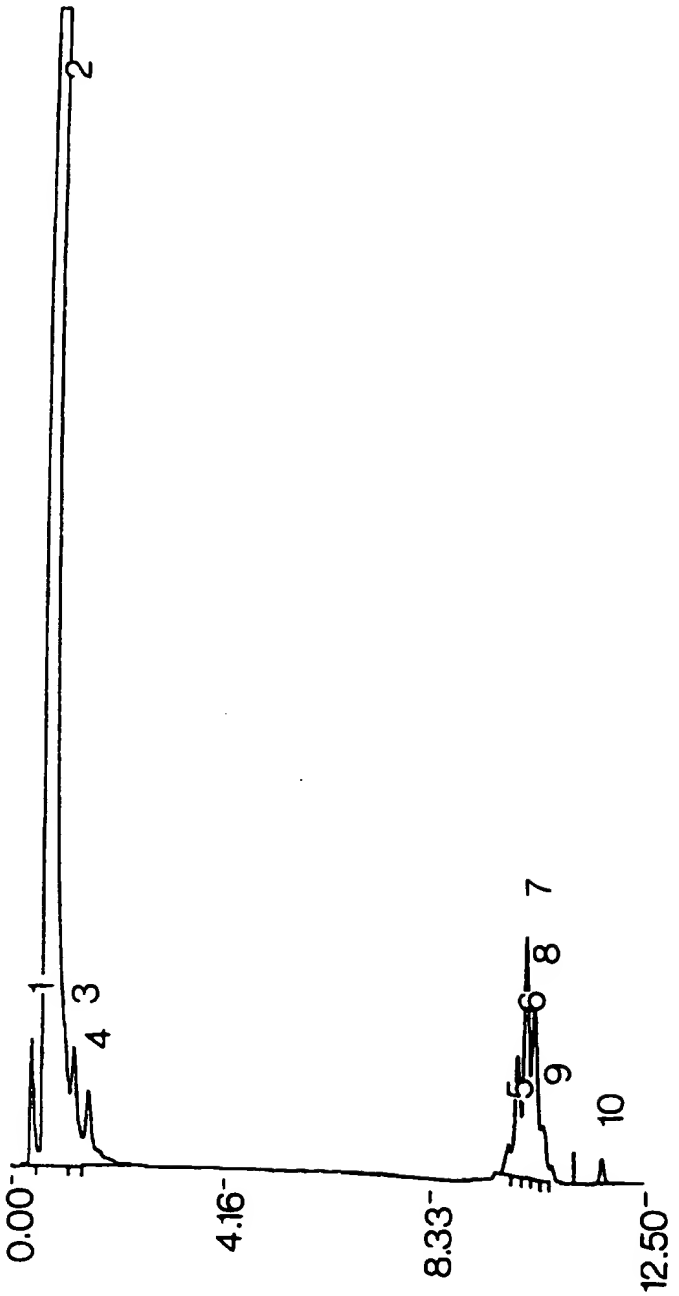
Graph 6a: Acetone supernatant of mycolic acids from *M. tuberculosis*, extracted with acetone after countercurrent purification



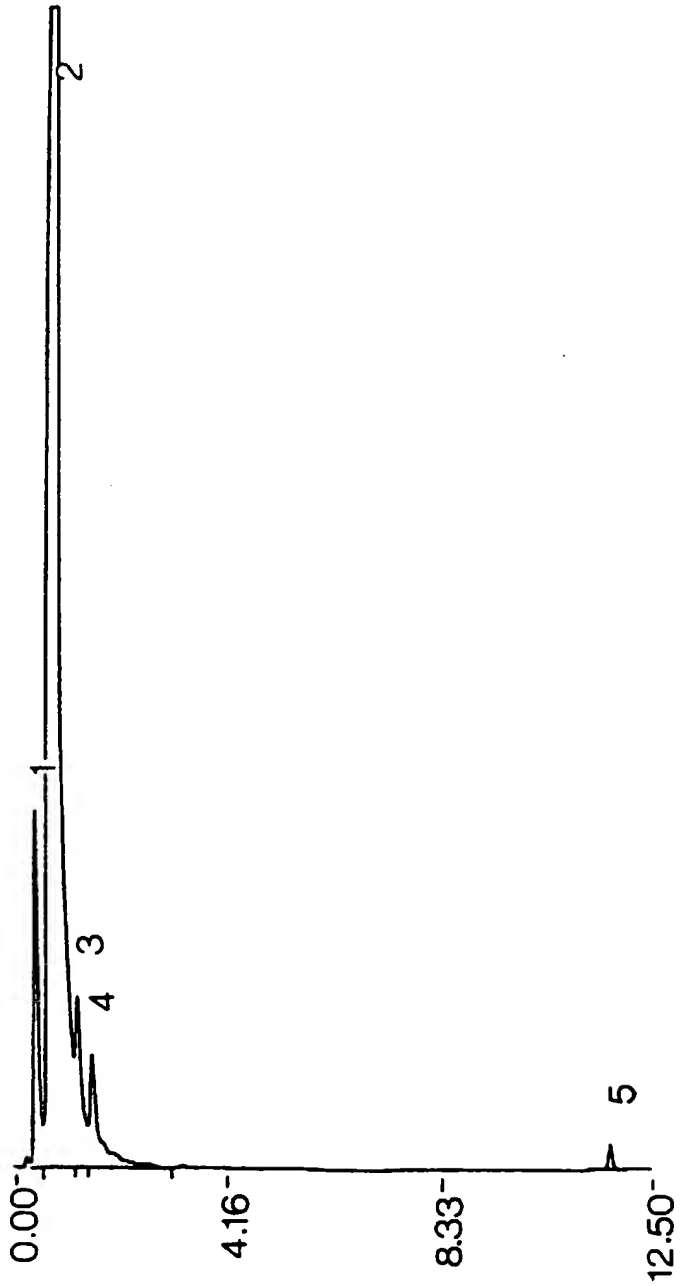
Graph 6b: Acetone supernatant of reagents extracted with acetone after countercurrent purification



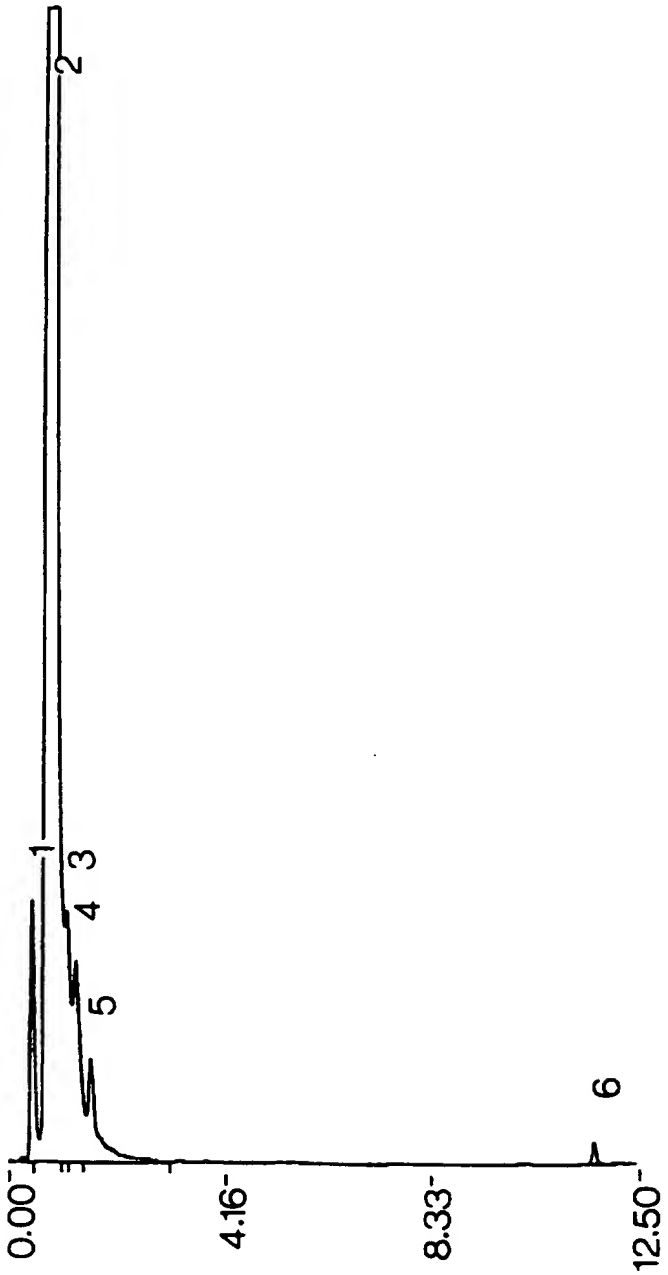
Graph 6c: Acetone supernatant of medium extracted with acetone after countercurrent purification



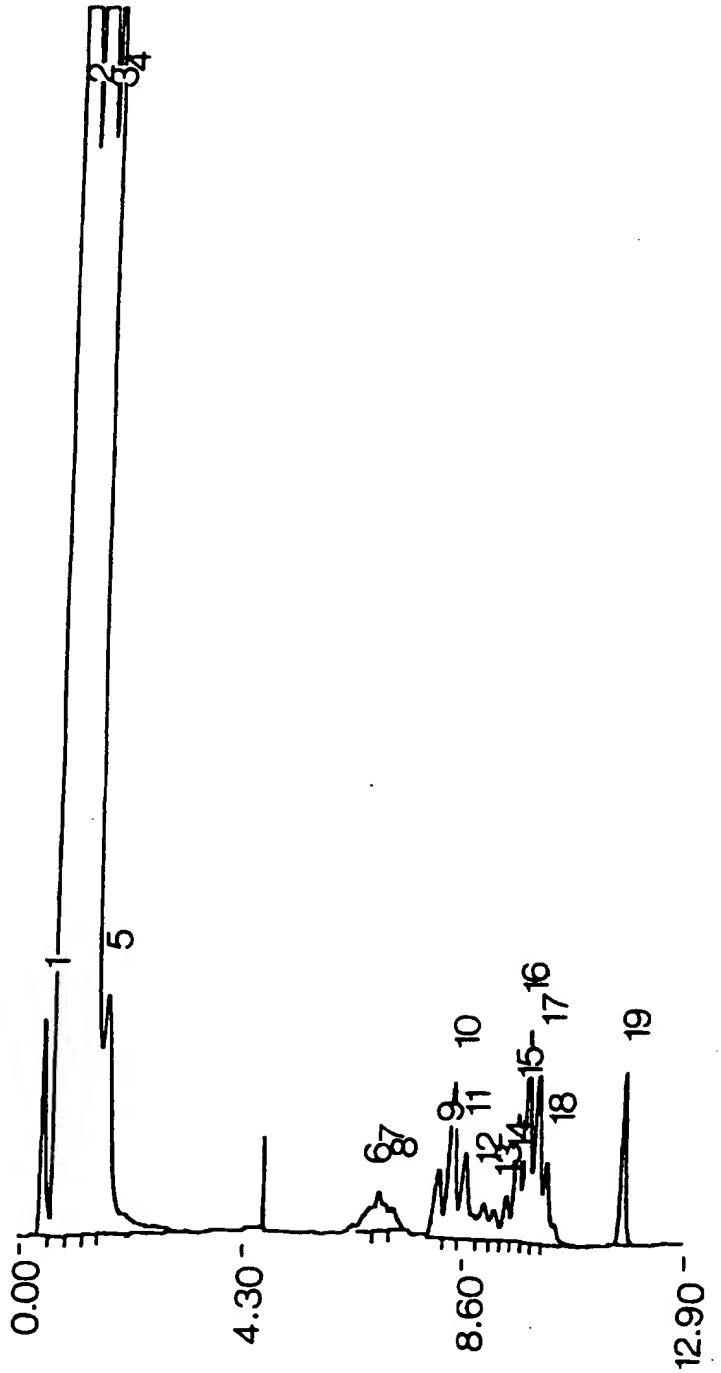
Graph 7a: Purified mycolic acids from *M. tuberculosis*, extracted with acetone after countercurrent purification



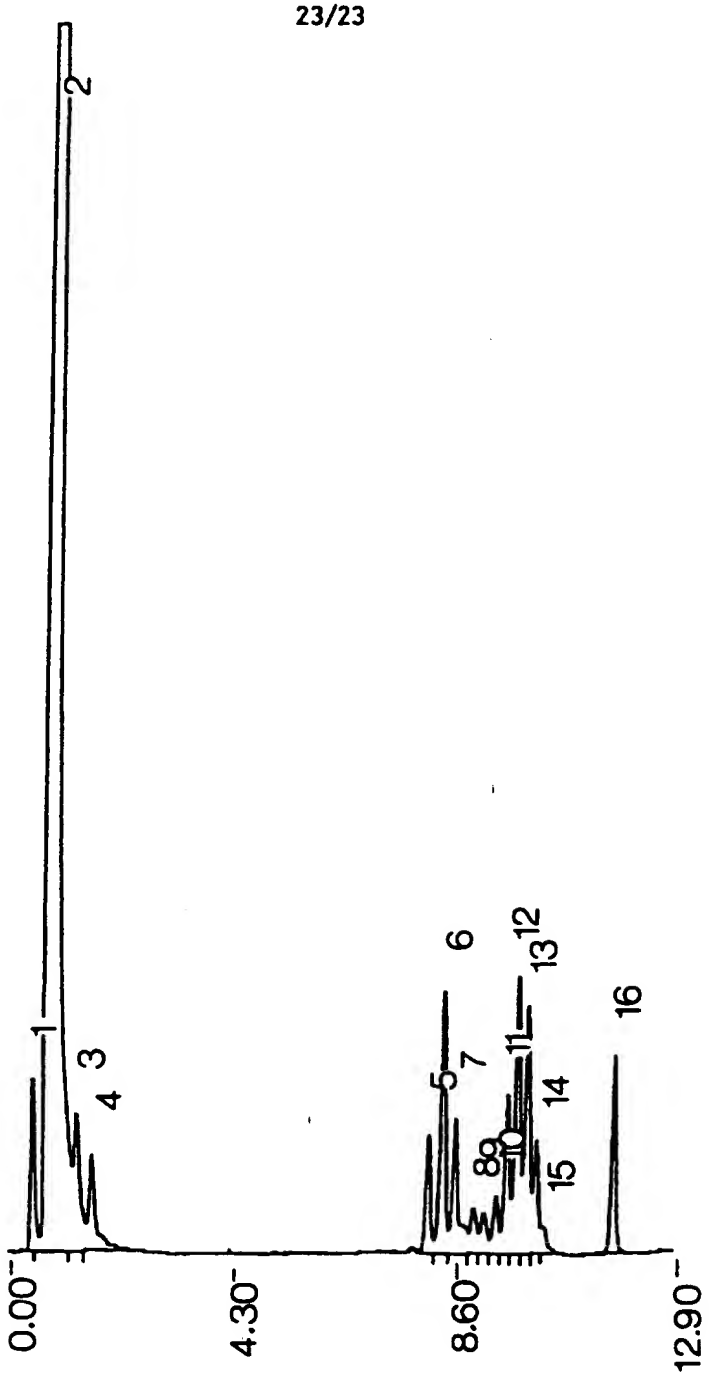
Graph 7b: Purified reagents, extracted with acetone after countercurrent purification



Graph 7c: Purified medium, extracted with acetone after countercurrent purification



Graph 8a: Crude extract from *M. vaccae*, resaponified



Graph 8b: Mycolic acids from *M. vaccae*, extracted with acetone, after countercurrent purification

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 96/00416

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12P7/64 A61K39/04 A61K35/74 C11B1/10 C07G17/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NATURE, vol. 372, 1994, LONDON GB, pages 691-694, XP002003831 BECKMAN E.M. ET AL: "Recognition of a lipid antigen by CD1-restricted alpha-beta+ T cells" see legend to figure 2 ("methods"); see legend to figure 3 ("methods") see page 692, column 1 ---	1-24
X	EP,A,0 261 248 (SAWAI PHARMACEUTICAL CO LTD) 1988 see example 1 ---	1-10, 12-15, 20-24
X	WO,A,95 00163 (BRIGHAM AND WOMEN'S HOSPITAL) 5 January 1995 see page 43 - page 48 ---	1-24
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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Date of the actual completion of the international search	Date of mailing of the international search report
24 May 1996	12.06.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fernandez y Branas,F

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INTERNATIONAL SEARCH REPORT

I· tional Application No
PCT/GB 96/00416

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO,A,95 28642 (ADCOCK INGRAM CRITICAL CARE LIMITED) 26 October 1995 see page 11 - page 26 see claims 8-17 -----	1-24

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INTERNATIONAL SEARCH REPORT

Information on patent family members

I· ational Application No
PCT/GB 96/00416

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-261248	30-03-88	WO-A-	8705606	24-09-87
		US-A-	5006514	09-04-91
WO-A-9500163	05-01-95	CA-A-	2165786	05-01-95
WO-A-9528642	26-10-95	AU-B-	2220595	10-11-95